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TITLE: Neural Resilience to Traumatic Brain Injury: identification of Bioactive Metabolites of Docosahexaenoic Acids Involved in Neuroprotection and Recovery

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13. SUPPLEMENTARY NOTES

During the entire award period, we tested the effects of n-3 fatty acids on the TBI recovery outcome. We first established a mouse model of TBI and dietary conditions to generate varying degrees of n-3 fatty acid deficiency. Using these models, we tested spontaneous recovery from TBI-induced motor and cognitive deficits in mice raised on n-3 fatty acid deficient diets. We found that extreme n-3 fatty acid deficiency where brain DHA was depleted by >70% significantly exacerbated TBI outcome for which a scientific publication in PLoS One was generated. Moderate depletion of DHA (by 30%) in the brain also showed the same trend; TBI-inflicted DHA-deficient mice exhibited slower functional recovery. Biochemical markers such as alpha spectrin cleavage, proinflammatory cytokines were elevated in the cortex of TBI-inflicted DHA-deficient mice which also showed less NeuN-positive neurons, resulting in a manuscript is in preparation. Both male and female mice were similarly affected by the DHA-depletion. An *in vitro* model to evaluate axon regeneration after injury has been established using cortical neuron cultures. Using this model we demonstrated the induction of axonal regrowth by synaptamide, a bioactive metabolite derived from DHA, for which a manuscript was prepared. We have also developed a method to identify DHA metabolites using stable isotope assisted mass spectrometry along with newly developed metabolite identification software, for which a manuscript is in preparation. Using quantitative mass spectrometry, the time course of the bioactive DHA-metabolite production in TBI-inflicted brain was determined. We identified three distinctive groups of metabolites according to the peak time of production after injury. The synaptamide level was steadily increased after TBI up to 48 h. We also observed improvement of TBI-induced motor deficit by directly injecting synaptamide following injury in FAAH KO mice. During the no cost extension period, we have made a significant progress in optimizing DHA administration protocol for the formation of bioactive metabolites such as synaptamide as well as functional recovery after TBI.

Table of Contents

Page

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Introduction

Military personnel in combat deployments are afflicted with high rates of traumatic brain injury (TBI) causing lifelong neurological and cognitive impairments, especially in learning and memory. Numerous studies have shown that docosahexaenoic acid (DHA) is essential for proper brain development and function [1,2], although the underlying mechanisms are still unfolding. Under normal conditions, DHA is present in esterified form in membrane phospholipids, especially the aminophospholipids, phosphatidylethanolamine (PE), and phosphatidylserine (PS). Despite tight regulation to maintain membrane phospholipid homeostasis, DHA enrichment can expand the PS pool in neuronal membranes [3], as DHA-containing phospholipids serve as the most favored substrate for PS biosynthesis in mammalian tissues [4]. On the contrary, depletion of DHA has been shown to decrease PS levels significantly in brain tissues [3, 5-7]. Since PS is known to participate in key signaling events supporting cell survival and differentiation, DHA-dependent PS modulation is an important aspect of neuroprotection [8]. Following brain injury, polyunsaturated fatty acids including DHA and arachidonic acid (AA, 20:4n-6) are released from neural membranes and metabolized to many bioactive derivatives. Some of the AA-derived eicosanoids are known to be pro-inflammatory, exacerbating the initial injury [9,10]. In contrast, some DHA-derived docosanoids has been shown to ameliorate or resolve inflammatory processes [11]. Furthermore, N-docosahexaenoylethanolamide (synaptamide), a DHA metabolite of a separate class, has been recently identified as a potent neuritogenic and synaptogenic agent [12]. In this regard, the DHA content of the brain may be an important variable to consider in devising a strategy to improve neuroprotection and recovery outcome after brain injuries.

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Objective

- The major goals of this project are to develop strategies to improve neural resilience to traumatic brain injury and facilitate recovery through mechanism-based optimization of the nutritional DHA or metabolite status in neuronal tissues.

- Aim 1. To determine if diets rich in DHA afford protection to the nervous system against traumatic brain injury in animal models
- Aim 2. To identify bioactive DHA metabolites formed in the brain that are involved in neuronal survival, neurite development, learning and memory
- Aim 3. To determine if DHA-derived mediators improve recovery after traumatic brain injury in animal models
- Aim 4. To devise therapeutic approaches for improving DHA status and/or administering specific bioactive metabolites that facilitate recovery from traumatic brain injury.

Statement of Work

Year 1

Task 1: Establishment of controlled cortical impact (CCI) traumatic brain injury model (months 1-8)
Task 2: Establishment of dietary condition to evaluate the neuroprotective effects of DHA (months 1-12 months)
Task 3: Setting up a dedicated LC/MS/MS instrument and metabolomics software, and analyzing DHA metabolites formed in cell culture and brain homogenates using the mass spectrometric approach coupled with stable-isotope labeling (1-18 months)

Year 2

Task 1: Testing effects of DHA status on TBI injury outcome using animal and dietary models established (months 9-24)
Task 2: Testing bioactivity of identified DHA metabolites in cell culture systems (months 13-24)
Task 3: Developing software in consultation with an instrumental company software team to establish a method to profile a broader range of DHA metabolites (months 6-18)

Year 3

Task1: Testing therapeutic potential of DHA and/or DHA metabolites administration on recovery after TBI (months 25-30)
Task 2: Analyzing active metabolites in the control and posttraumatic brains during the course of recovery after dietary manipulation of DHA status or DHA administration (months 18-36)
Task 3: Optimizing the DHA administration protocol (months 31-36)

Year 4 (No-cost Extension)

Task 1: Testing effects of DHA status on CHI injury outcome using animal and dietary models established (months 9-24)
Task 2: Testing therapeutic potential of DHA and/or DHA metabolites administration on recovery after TBI (months 25-30)
Task 3: Optimizing the DHA administration protocol (months 31-36)

Report

Year 1

Task 1: Establishment of controlled cortical impact (CCI) traumatic brain injury model (months 1-8)

During this report period, we have successfully established the Controlled Cortical Impact TBI model and the Animal Study Proposal (ASP) to perform traumatic brain injury research has been approved by NIH ACUC and ACURO.

The TBI model was set up using the Traumatic Brain Injury Device (*Precision Systems and Instrumentation*) and a sterotaxic frame (*Kopf*). The device includes an impactor tip of 3 mm that inflicts injury to the exposed brain by striking it directly. The velocity of the impact, the depth of the impact and the dwell time for the impact were standardized to have reproducible injury. The functional outcome was assessed by the accelerating rotarod test. The extent of the injury was assessed by direct visualization of the injured area by histology. Cresyl violet staining was used to stain the injured brain sections and to inspect the size of the cavity resulting from traumatic brain injury.

For the accelerating rotarod test, the mice were pre-trained to the rotarod apparatus for three days and a baseline reading of the mice was recorded on the day prior to the surgery. The rotarod tests were further performed from the first day after injury and each day till the fourth day to assess their performance after the injury as well as during spontaneous recovery.

As assessed by the rotarod test, the motor deficits were transient in nature and restored later in time. The 1.5 mm impact group showed greater deficits than the 1mm impact group and exhibited a significant reduction in motor performance as compared to the sham group on the first three days after surgery ($p<0.05$). The deficit was significant for the 1mm impact group only on the second day after surgery. The deficit observed for the 1.5 mm group was significantly different from that observed for the 1 mm group on the 1st day after the surgery.

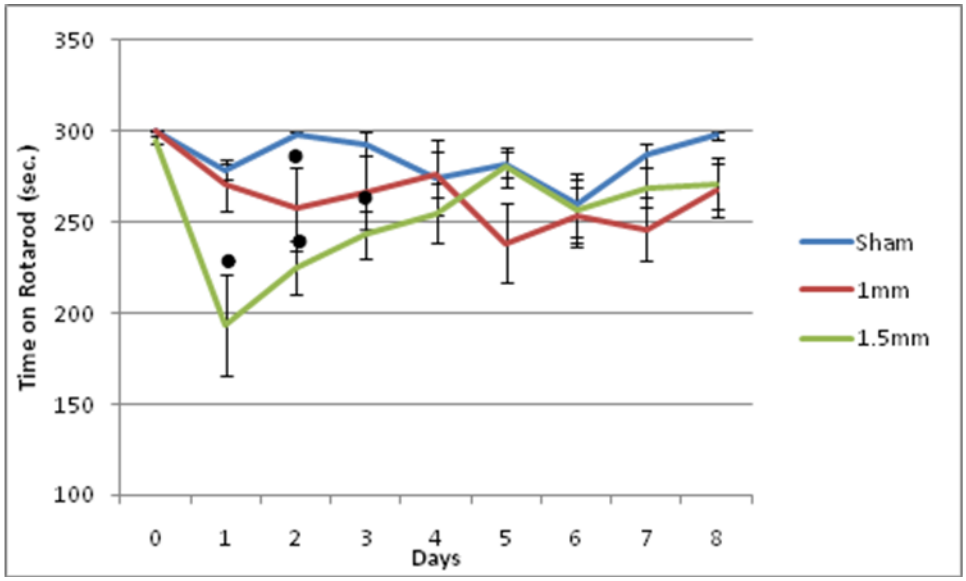


Fig.1: Rotarod performance of mice in sham, 1mm impact depth and 1.5mm impact depth TBI groups (• designates $p<0.05$).

The brains of mice subjected to TBI were removed after euthanization on the eighth or ninth day after surgery. Frozen sections of the brains were obtained using a cryotome. At least 4 equidistant sections were taken for each brain. They were stained with cresyl violet. The cavity in the ipsilateral cerebral hemisphere was measured using the Metamorph software and expressed as the percent of the ipsilateral hemispheric area. An increase in brain injury was observed with increasing impact depth. Based on our experience during the pilot studies, an impact depth of 1.5mm was selected for further studies. The rest of the impact parameters remained constant. The animal study proposal (ASP) to perform traumatic brain injury research using this procedure has been approved by NIH ACUC and ACURO.

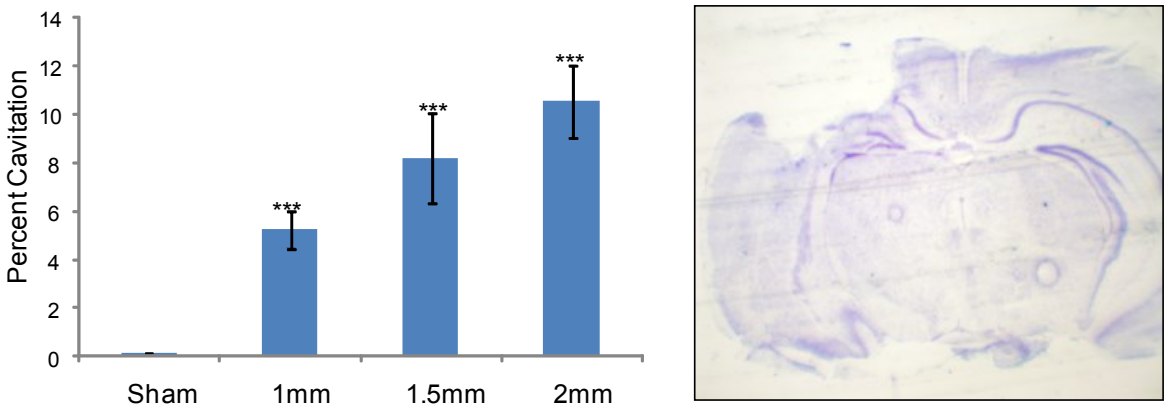


Fig. 2: Brain cavitation in mice subjected to TBI (expressed as percent of the ipsilateral hemisphere). ***, $p<0.001$ as compared to sham.

Additional behavioral tests have been established for refined evaluation of the degree of deficit and the response to therapeutic intervention; the novel object recognition test for cognitive deficit and the beam walk test for confirmation of the motor deficit (Fig. 3). For the novel object recognition test, mice were individually acclimatized to the test arena daily for 10 min for three days. On the third day, the animals were exposed to two objects for 10 min each and subsequently tested for memory after 2 hours. For the beam walk test, the mice were trained to traverse a narrow beam before the surgery and the number of hind foot slips was observed after surgery and compared to sham operated animals. Further refinement of the test parameters is currently in progress.

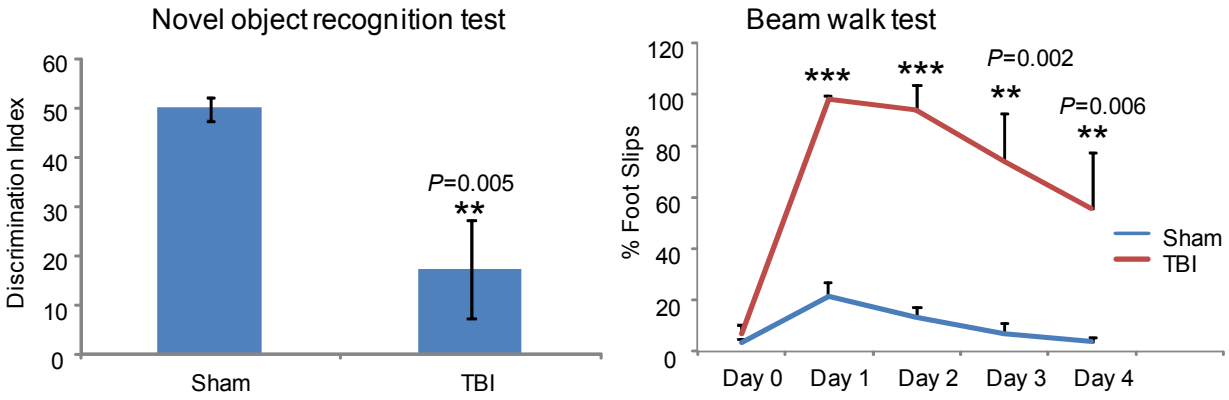


Fig. 3: Novel object recognition test and beam walk test performance of mice in sham and TBI groups. ***; $p<0.001$ as compared to sham.

Task 2: Establishment of dietary condition to evaluate the neuroprotective effects of DHA (months 1-12 months)

During this report period, we have successfully established a dietary condition to generate extreme n-3 fatty acid depletion in the mouse brain. Furthermore, we were able to obtain preliminary results regarding the effects of DHA status on the recovery from TBI-induced motor deficit using the CCI-model established during this period.

To test whether the DHA status influences the spontaneous recovery potential after TBI, we have first generated extreme cases of DHA-deficiency by rearing mice on an omega-3 deficient diet for three generations as compared with the adequate diet group. Pregnant C57/BL6 mice from Charles River Laboratories (Portage, MI) were fed with either an n-3 fatty acid adequate or deficient diet throughout the pregnancy and lactation period. The second generation offspring females were continued on each respective diet and bred for the third generation. Both semi-synthetic pelleted diets were based on the AIN-93G formula (Reeves et al., 1993) varied only in fat composition (Dyets, Bethlehem, PA). The adequate diet consisted of 7.45, 1.77, 0.48 and 0.3 g of hydrogenated coconut, safflower, flaxseed and DHASCO oil in 100g of diet. The deficient diet contained 8.1 and 1.9 g of hydrogenated coconut and safflower oil per 100g. The resulting n-3 fatty acid content was 2.5 wt% LNA (18:3n-3) plus 0.9 wt% DHA in the adequate diet, and only 0.09 wt% of LNA in the deficient diet. The procedures employed in this study were approved by the National Institute on Alcohol Abuse and Alcoholism (LMS-HK21).

The brain DHA level in the third generation n-3 deficient animals was lowered by over 70%, which was mostly compensated by the increase of docosapentaenoic acid (DPA, 22:5n-6). Age and gender matched mice at 10-12 weeks from adequate and deficient groups were subjected to the CCI procedure established in this laboratory and the TBI-induced motor deficit was evaluated. Preliminary results indicated a significant difference in spontaneous recovery (Fig. 4). The motor function of omega-3 adequate animals was recovered by day 2 after TBI. In contrast, the deficient group showed prolonged motor deficit until day 4 after TBI. Statistical significance between two groups has been already observed with only with 4 animals from each group. Further tests with more animals are being performed to evaluate both motor and cognitive deficit, as deficient and adequate animal pairs become available.

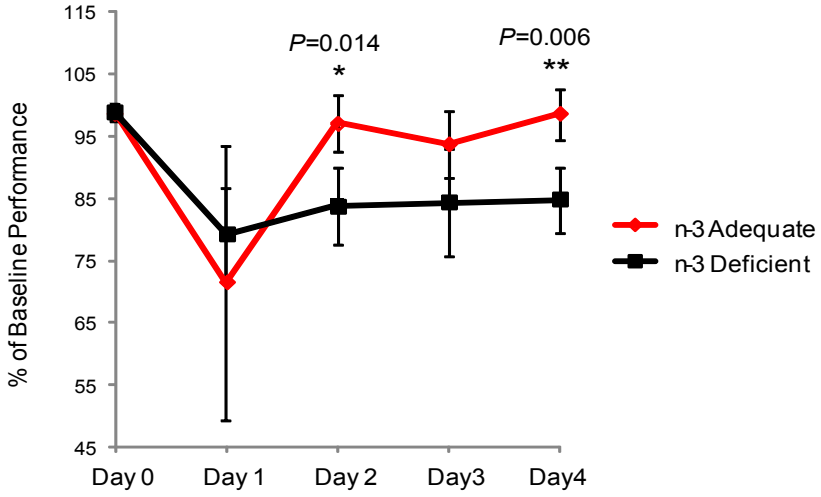


Fig. 4: Effects of dietary n-3 fatty acids on spontaneous recovery of TBI-induced motor deficit evaluated by rotarod test (n=4 for each group).

Task 3: Setting up a dedicated LC/MS/MS instrument and metabolomics software, and analyzing DHA metabolites formed in cell culture and brain homogenates using the mass spectrometric approach coupled with stable-isotope labeling (1-18 months)

Due to delayed gifting process, the purchase of the Q-Exactive mass spectrometer (MS) was substantially delayed. The order has been placed only in late January of 2012, and after the usual 60 days of NIH central procurement process the purchase order has been just awarded to the company. This high resolution MS is expected to arrive in the middle of May and the installation and evaluation process will take a month or two. Once the instrument is properly installed, we will be able to search for new DHA metabolites formed in cell cultures and brain homogenates. Despite the delay, we were able to establish analytical methods to profile known metabolites of arachidonic acid (AA) and DHA in TBI brains using existing LC/MS/MS during this period. In parallel, we established the cortical cell culture and identify the formation of the major DHA metabolite we have previously observed from hippocampal neuronal culture. The cortical neuronal cultures were chosen for the bioactivity test planned to be performed once additional DHA metabolites are identified, since the cortical region would be most significantly inflicted by the CCI procedure. The effects of DHA and N-docosahexaenoylethanolamide (synaptamide), a potent neuritogenic and synaptogenic DHA metabolite, have been subsequently characterized in cortical cultures.

In preparation for the analysis of TBI-induced metabolites from mouse brains with different DHA status, we have established a metabolite profiling method using the multiple reaction monitoring mode (MRM) by electrospray LC/MS/MS. Using this method, we first characterized the TBI-induced production time course for the endogenous metabolites of AA and DHA. During the first 24h, PGE2, PGD2, 12-hydroxyeicosatetraenoic acid (12-HETE) and 14-hydroxydocosahexaenoic acid (14-HDHE) were significantly increased in the TBI-inflicted brains (Fig. 5). We also observed that synaptamide level was significantly elevated by 24h after TBI in comparison to the sham operated controls.

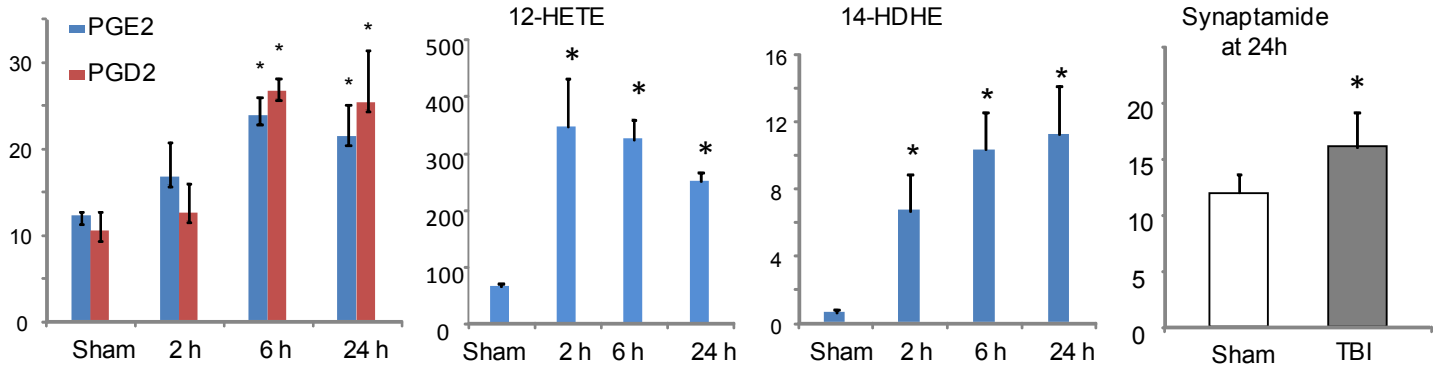


Fig. 5: Time course of metabolite formation after TBI. Y axis unit: pmol/g tissue
*, $p<0.05$ as compared to sham

Formation of synaptamide was also observed in cortical cultures supplemented with DHA. As with the hippocampal neurons, cortical neurons responded to DHA and synaptamide, to the latter with significantly higher sensitivity. Neurite growth of cortical neurons was promoted by DHA and synaptamide dose-dependently while oleic acid (OA) did not exert any effects (Fig. 6). Formation of additional DHA metabolites in the cortical cultures will be examined using the stable isotope labeling assisted metabolomics approach once the high resolution MS is installed.

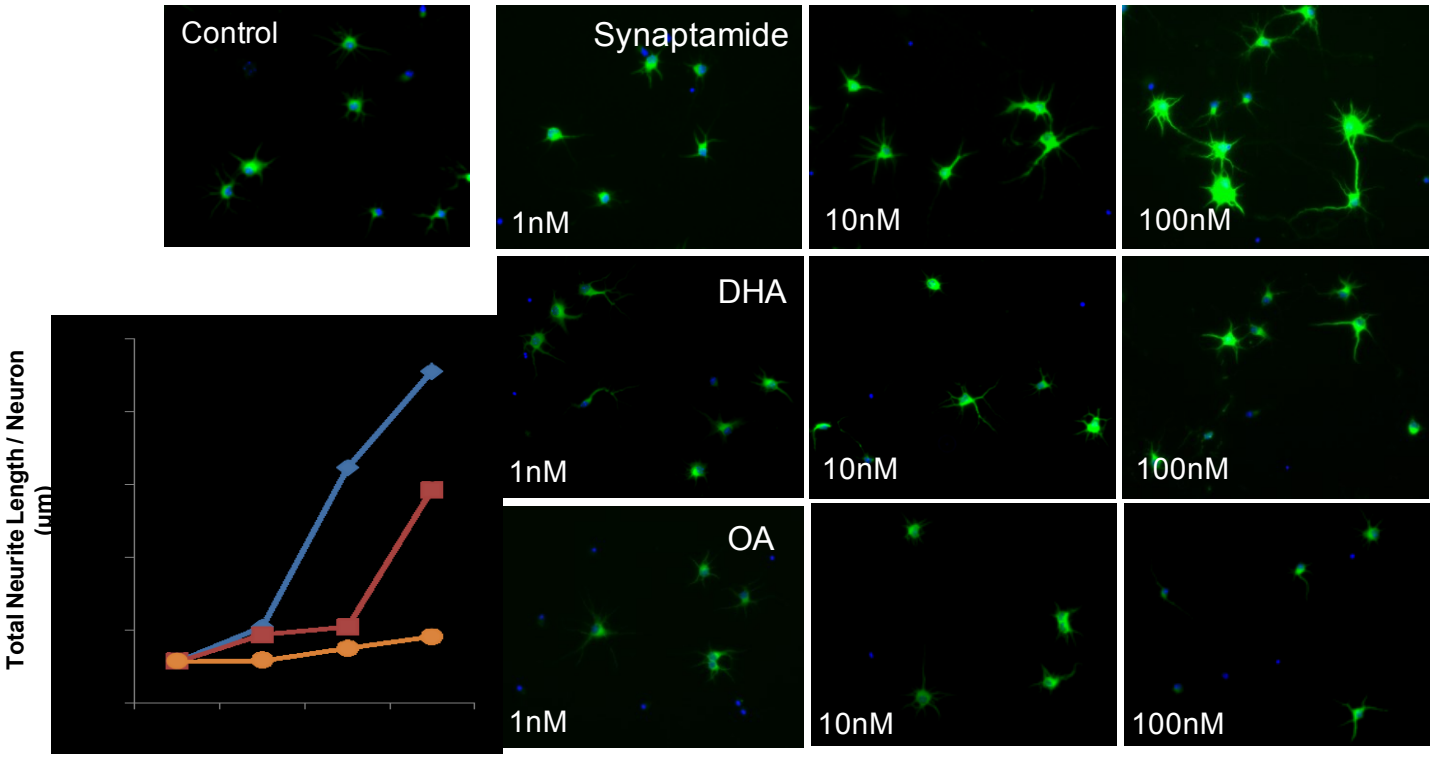


Fig. 6: Dose-dependent increase of cortical neurite outgrowth by DHA and synaptamide.

Since synaptamide is a potent synaptogenic agent and its production is elevated after TBI, we tested whether this compound can assist the recovery process after TBI. Because synaptamide, like anandamide, is a good substrate for fatty acid amide hydrolase (FAAH), we have tested the effects of synaptamide in FAAH KO mice by i.p. administration at 15 min, 24h and 48h after TBI. The preliminary results indicated that the motor deficit evaluated by the rotarod test was significantly improved at 24 and 48h after TBI (Fig. 7), suggesting therapeutic potential of synaptamide for functional improvement after TBI.

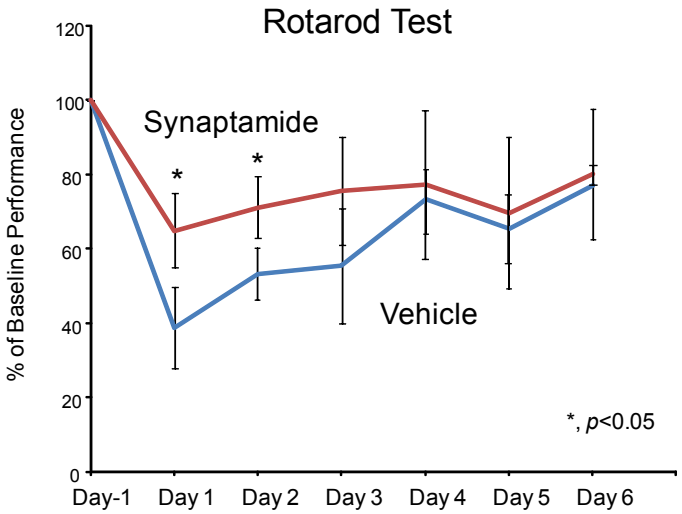


Fig. 7: Improvement of TBI-induced motor deficit by synaptamide injection (10 mg/kg, i.p.) in FAAH KO mice (n=6 for each group). *, p<0.05 vs. Vehicle group.

Year 1 Key Research Accomplishments

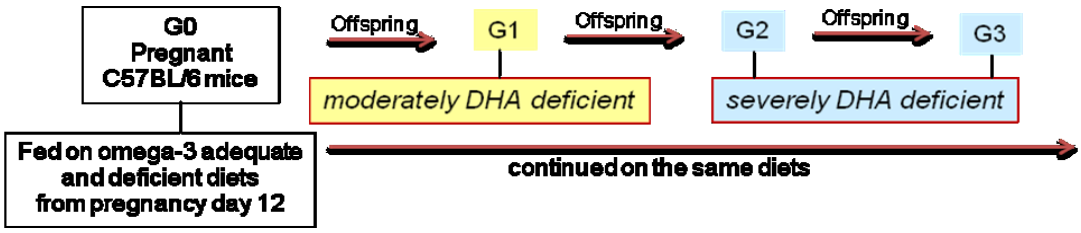
1. A mouse model of TBI has been established although further refinement of behavioral test parameters to evaluate the functional deficit is still in progress.
2. TBI-induced formation of endogenous DHA/AA metabolites including synaptamide, a previously characterized neuritogenic and synaptogenic derivative of DHA, has been identified in mouse brains.
3. A dietary condition to generate extreme n-3 fatty acid depletion in the mouse brain has been established for testing TBI outcome.
4. Cortical neuronal culture has been established.
5. DHA metabolism to synapamide has been identified in cortical neuronal cultures.
6. Neuritogenic effects of DHA and synaptamide have been confirmed in cortical cultures.
7. Preliminary results:
   a. Significant retardation of spontaneous recovery from TBI-induced motor deficit has been observed in mice raised on an n-3 deficient diet where brain DHA content was depleted by over 70%.
   b. Improvement of TBI-induced motor deficit by synaptamide injection following injury has been observed in FAAH KO mice.

Year 2

Task 1: Testing effects of DHA status on TBI injury outcome using animal and dietary models established (months 9-24)

During this report period, we established the effects of DHA status on the recovery from TBI-induced motor deficit using the CCI-model established during the last period. We also established moderately DHA-deficient model in addition to the extreme DHA-deficient model.

During the last report period, we have successfully established the CCI-model for TBI. During this period, we further refined behavioral test parameters to evaluate the functional deficit associated with TBI. We also established dietary conditions to generate moderate and extreme DHA depletion in the mouse brain by feeding mice with an omega-3 deficient special diet for one to three consecutive generations (G1-G3) (Scheme 1). The brain DHA level in the second or third generation omega-3 deficient animals was lowered by over 70%, which was mostly compensated by the increase of docosapentaenoic acid (DPA, 22:5n-6) (Fig. 1).



Scheme 1. Generation of moderate and severely DHA-deficient mice

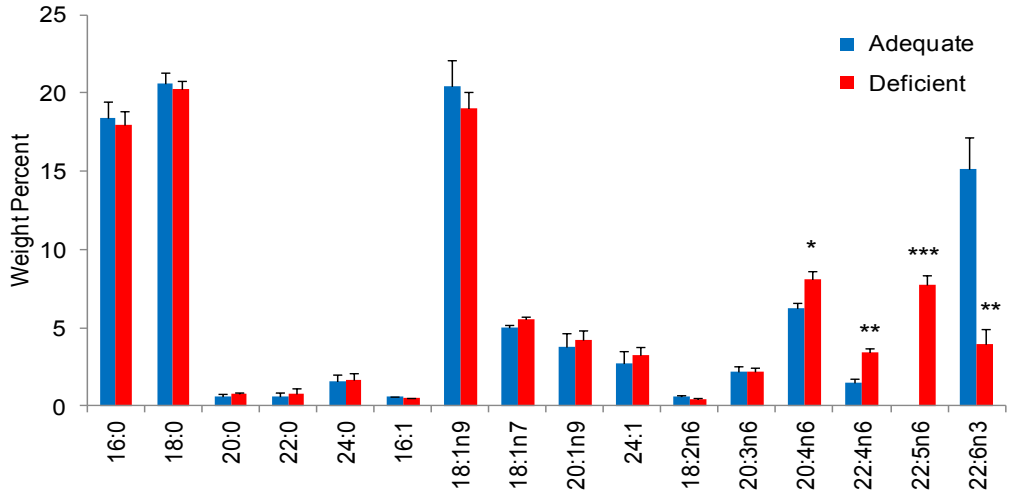


Fig. 1. Fatty acid composition of the brains from omega-3 fatty acid adequate and severely deficient mice. Data are expressed as mean ± SD (n=3 for each group). *p<0.05, **p<0.01, and ***p<0.001 compared to the adequate group

Using these animal models, we investigated the effect of DHA-depletion on the TBI outcome. Age and gender matched mice at 10-12 weeks from adequate and severely deficient groups were subjected to the CCI procedure and the TBI-induced motor and cognitive deficits were evaluated using accelerating rotarod, beam walk and novel object recognition (NOR) tests. For the accelerating rotarod test, the mice were pre-trained for the rotarod apparatus for three days and a baseline reading of the mice was recorded on the day prior to the surgery. For the beam walk test, the mice were trained to traverse a narrow beam before the surgery and the number of hind foot slips was observed after surgery and compared to sham operated animals. The rotarod and beam walk tests were further performed from the first day after injury and each day during spontaneous recovery until day 7 after TBI. The two diet groups showed a significant difference in spontaneous recovery of motor function (Fig. 2). By day 3 after TBI the rotarod and beam walk performances of omega-3 adequate animals was recovered significantly. In contrast, the deficient group showed prolonged motor deficits until day 7 after TBI. Statistical significance between two groups was reached with 8 animals from each group.

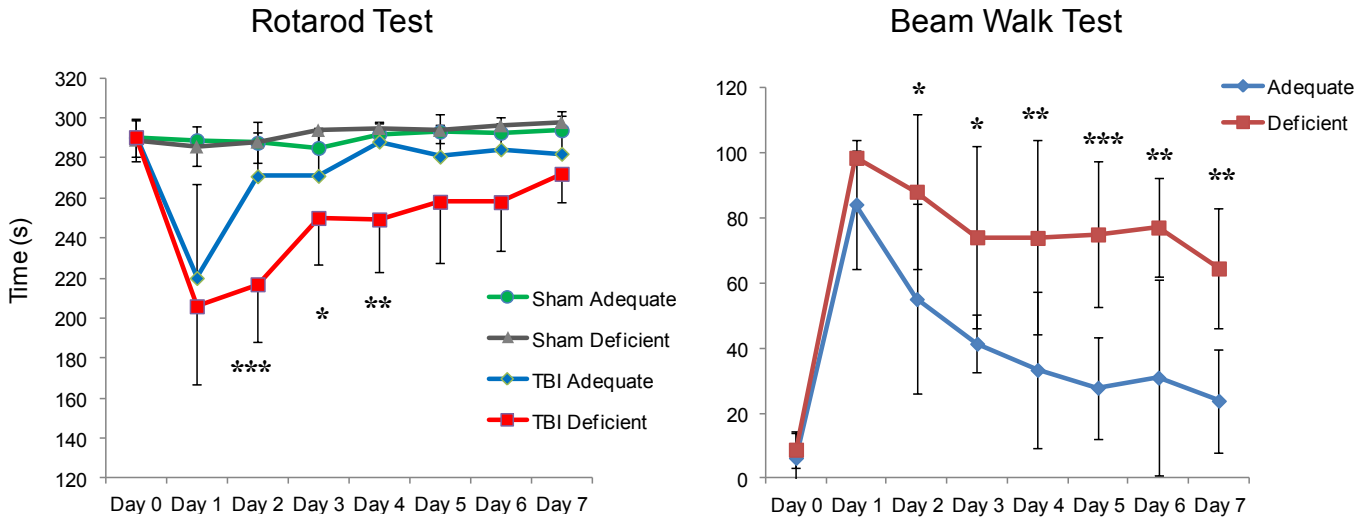


Fig. 2. Effects of dietary omega-3 fatty acids on spontaneous recovery of TBI-induced motor deficits evaluated by rotarod and beam walk tests. Data are expressed as mean ± SD (n=8 for each group). *p<0.05, **p<0.01, and ***p<0.001 as compared to the TBI adequate group.

The novel object recognition (NOR) test was performed on day 7 after TBI. Mice were individually acclimatized to the test arena daily for 10 min for three days prior to the testing. On the third day, the animals were exposed to two objects for 10 min each and subsequently tested for memory after 2 hours. In sham animals, the NOR performance between omega-3 adequate and severely deficient groups was not statistically different. Nevertheless, NOR performance was significantly impaired in TBI-inflicted severely omega-3 deficient animals compared to the corresponding adequate group (Fig. 3). This data suggests that DHA-depleted animals are particularly susceptible to TBI-induced cognitive deficit and adequate omega-3 fatty acid provision may have a role in preventing such deficit.

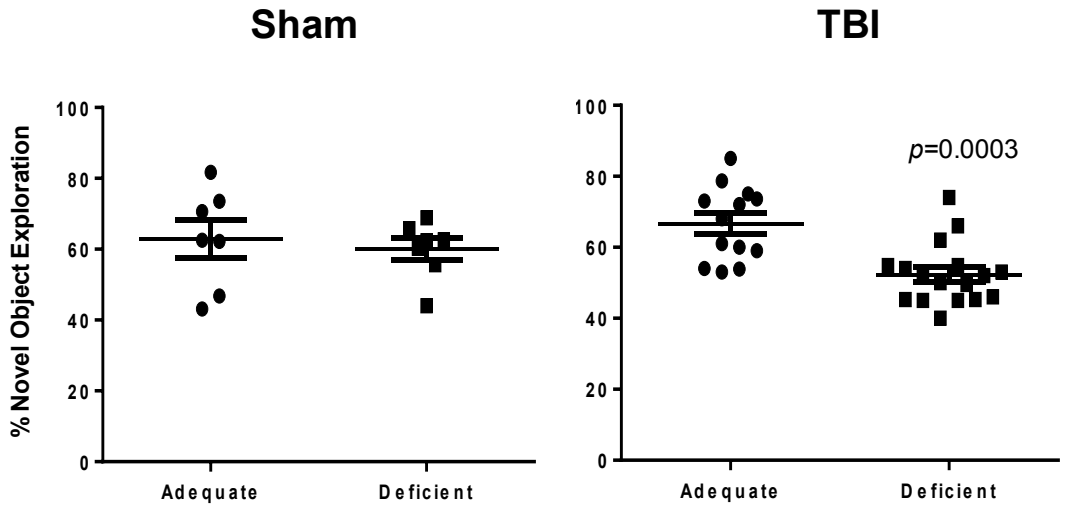


Fig. 3. Effects of dietary omega-3 fatty acids on the TBI-induced cognitive deficit evaluated by novel object recognition test. Data are expressed as mean ± SE (n=7-17 for each group). *p*= 0.0003 as compared to the TBI adequate group.

We have also evaluated the anxiety-like behavior using open field test (Fig. 4). Animals with severe omega-3 deficiency showed significantly less time in the center zone compared to the adequate animals, indicating that DHA-deficiency alone can cause increased anxiety. TBI significantly increased anxious behavior in both adequate and deficient groups; however, the significant difference between two groups remained after TBI. These data suggest that the TBI-induced anxious behavior can be exacerbated by severe omega-3 deficiency.

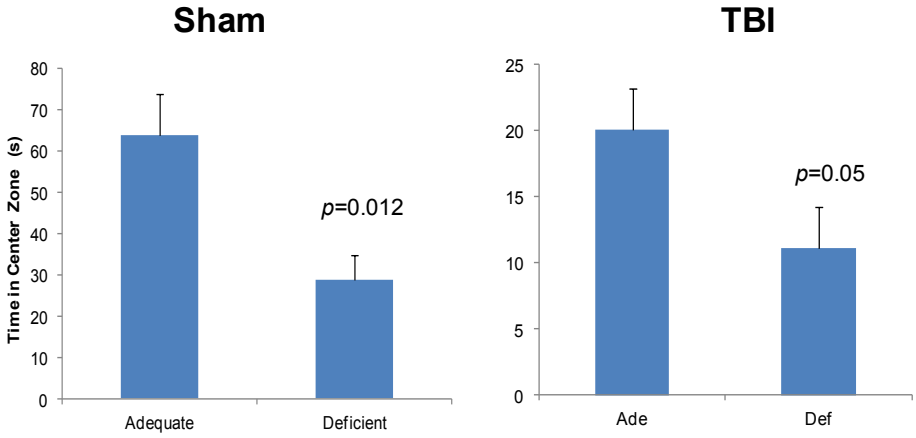


Fig. 4. Effects of dietary omega-3 fatty acids on the TBI-induced anxious behavior evaluated by open field test. Data are expressed as mean ± SD (n=7-8 for each group). *p*= 0.012 and 0.05 as compared to the corresponding TBI adequate group.

Upon completion of the behavioral tests, brains were harvested (on day 8 after TBI), and histological and biochemical evaluation was performed including cavity size, NeuN staining and western blotting for spectrin

alpha. Although statistical insignificance was not reached, the cavity size of the TBI-inflicted deficient brains showed an increasing trend compared to the adequate group (Fig. 5). Western blot analysis indicated that degradation of alpha spectrin, a marker of TBI induced injury, was elevated in the cortex of TBI-inflicted DHA-deficient mice after 24 h of injury (Fig. 6). Immunohistochemical analysis of NeuN, a neuronal marker protein, showed a reduction of NeuN-positve cells in the injured hemisphere in comparison to uninjured hemisphere (40-60% reduction). The NeuN positive cells in the injured hemisphere were further decreased in DHA-depleted brains in comparison to the DHA-adequate brains (Fig. 7). While *p* value was 0.06, only 3 animal samples have been tested so far, and it is likely that statistical significance will be reached when additional samples are analyzed. These biochemical and histological results consistently supports the behavioral outcome where omega-3 adequate mice recover better than deficient animals.

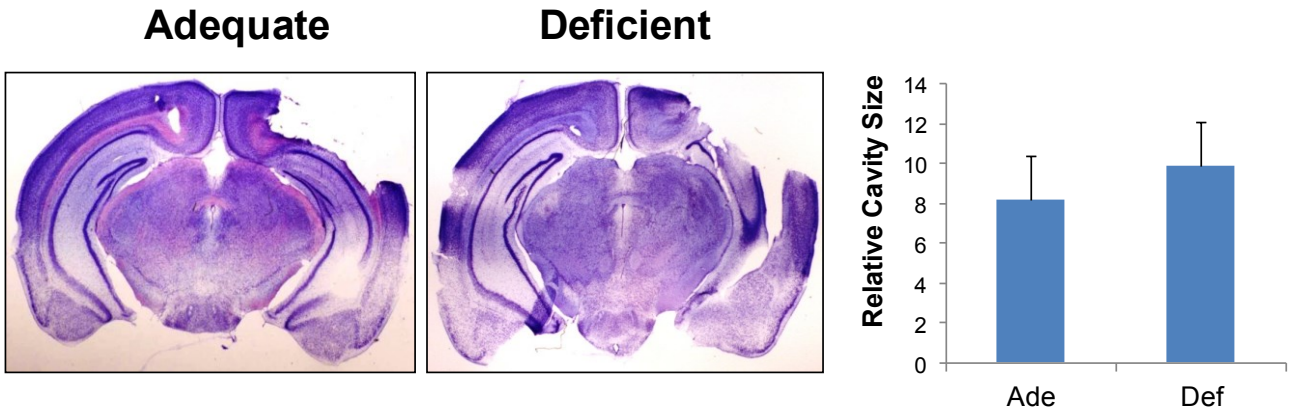


Fig. 5. Cresyl violet staining of brain sections and quantification of brain cavities in TBI-inflicted omega-3 fatty acid adequate and severely deficient mice. Data are expressed as mean ± SD (n=5 for each group).

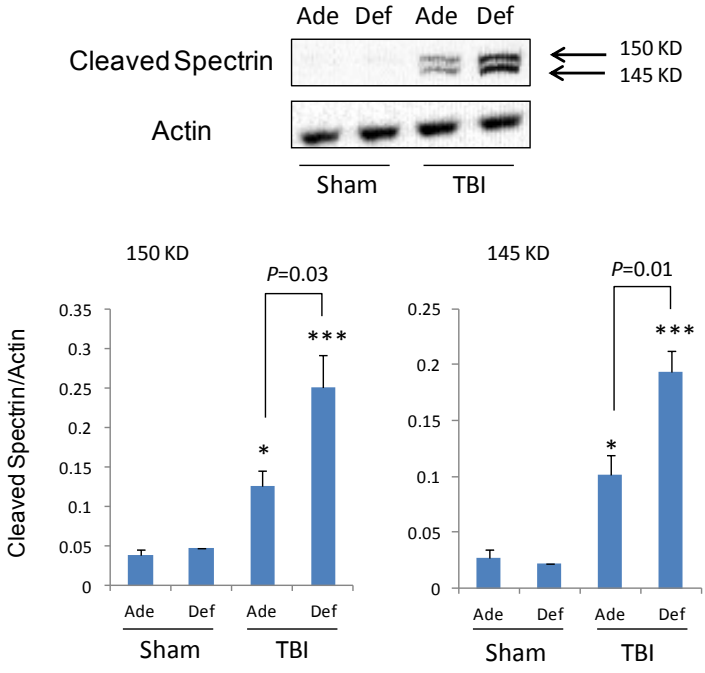


Fig. 6. Western blot analysis of cleaved spectrin in TBI-inflicted omega-3 fatty acid adequate and severely deficient mouse brains. Data are expressed as mean ± SD (n=4 for each group).

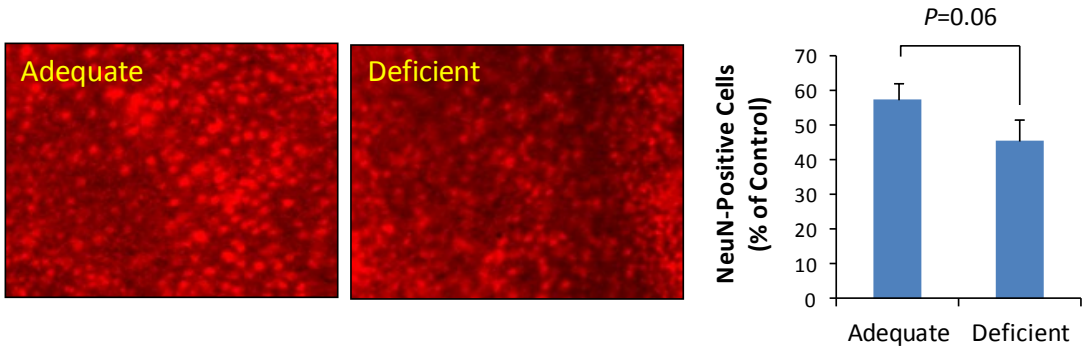


Fig. 7. NeuN immunostaining of brain sections and quantification of NeuN-positive cells in TBI-inflicted omega-3 fatty acid adequate or severely deficient mice. % of NeuN-positive cells in the injured hemisphere was calculated against NeuN-positive cells in the uninjured hemisphere. Data are expressed as mean ± SD (n=3 mice with 3 sections/mouse for each group).

Using the extreme case of DHA depletion, we established that DHA-adequate mice recover better from TBI compared to the DHA-deficient mice. We have extended this study to a model of moderate DHA deficiency which commonly occurs in humans. The moderately DHA-depleted mice (G1) were generated by feeding pregnant mice a special omega-3 deficient diet from the gestation day 12 (Scheme 1) throughout pregnancy and lactation period and offspring mice were continued on the same diet until the time of experiments (10-12 weeks old). Such deprivation of dietary omega-3 fatty acids effectively lowered brain DHA by 30% compared to the omega-3 adequate group (Fig. 8).

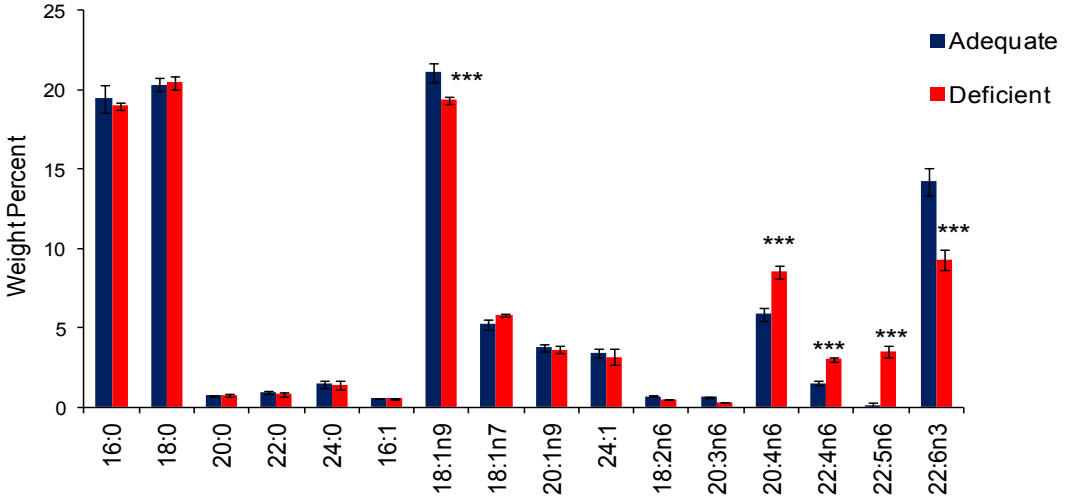


Fig. 8. Fatty acid composition of the brains from the first generation offspring mice fed on omega-3 fatty acid adequate and deficient diets. Moderate DHA-depletion was induced. Data are expressed as mean ± SD (n=3 for each group). ***p<0.001 compared to the adequate group.

Moderate DHA-depletion also had a similar impact on the TBI outcome, although the extent was not as severe as the extreme case of DHA-depletion. Both rotarod and beam walk tests indicated significant differences between adequate and deficient groups with the latter showing slower recovery (Fig. 9). In addition, cognitive function and anxious behavior were also adversely affected by the moderate omega-3 fatty acid deficiency in TBI-inflicted mice (Fig. 10).

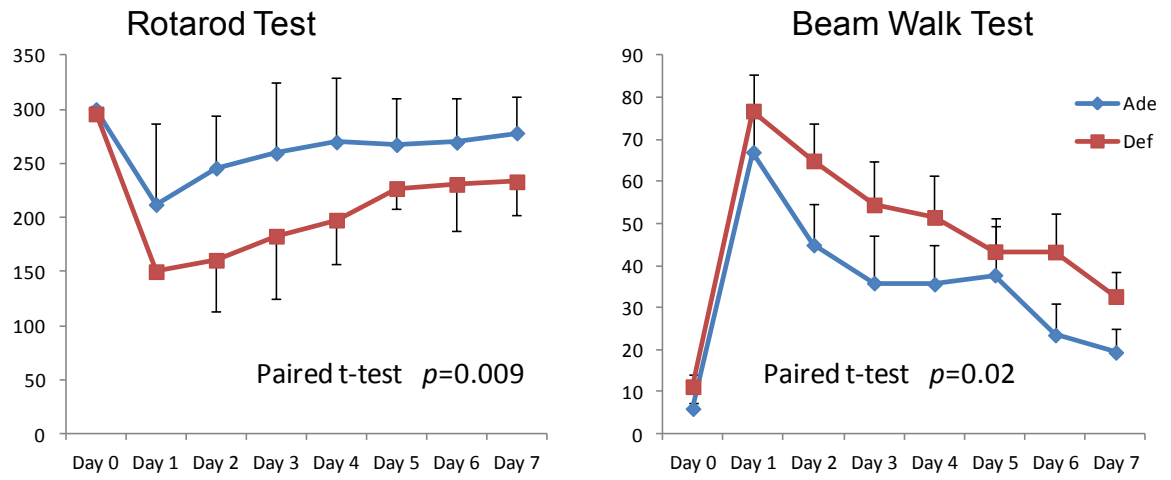


Fig. 9. Effects of moderate omega-3 fatty acid deficiency on spontaneous recovery of TBI-induced motor deficits evaluated by rotarod and beam walk tests. Data are expressed as mean ± SE (n=8 for each group). Paired t-test indicates significant difference between two groups.

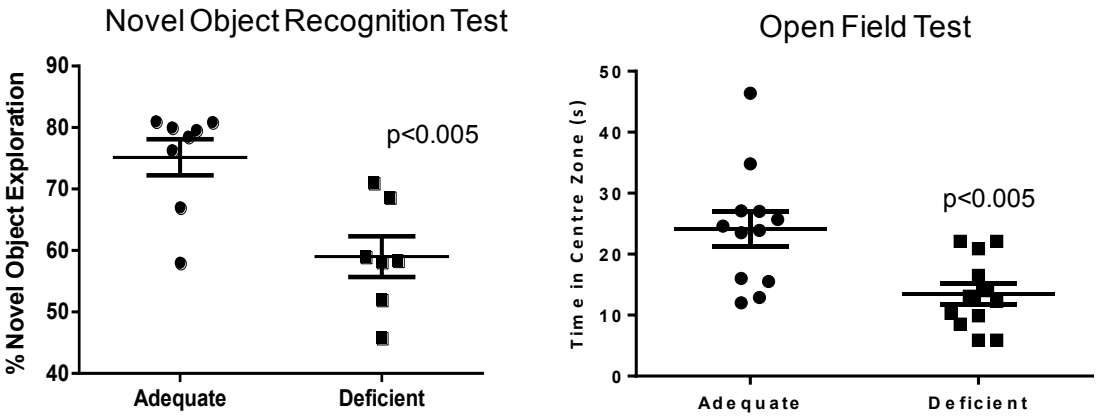


Fig. 10. Effects of moderate omega-3 fatty acid deficiency on the cognitive function and anxiety in TBI-inflicted mice evaluated by novel object recognition and open field tests, respectively. Data are expressed as mean ± SE.

Our results indicated that the mice from both severe and moderate DHA-deficiency groups had slower recovery of vestibulomotor functions as assessed by the rotarod and beam walk tests. Unlike the n-3 adequate controls, the brain injured n-3 deficient mice also failed to discriminate between the familiar and novel objects in the object recognition test, indicating impaired memory. Histological analysis revealed an increased cavity volume in the n-3 deficient group along with less NeuN-positive neurons, indicating exacerbated injury. Western blot analysis indicated that degradation of alpha spectrin, a marker of TBI induced injury, was elevated in the cortex of TBI-inflicted DHA-deficient mice after 24 hours of injury. These results consistently indicate that DHA-adequate mice recover better from TBI. Considering that modern diets have low n-3 fatty acids and DHA deficiency is common in humans, results from this study present strong possibility of using nutritional remediation as a tool to enhance recovery from brain injury.

Task 2: Testing bioactivity of DHA metabolites in cell culture systems (months 13-24)

We have previously identified N-docosahexaenoylethanolamide (synaptamide) as a potent neuritogenic and synaptogenic metabolite of DHA formed in the hippocampal and cortical neuronal cultures. In addition to dendrite extension, we tested the bioactivity of synaptamide on axon growth in cortical neurons during this

period. The cortical neuron cultures were chosen, since the cortical region would be most significantly inflicted by the CCI procedure.

Axon growth of cortical neurons was promoted by DHA dose-dependently in the 0.1-1 μM range. DHA-derived synaptamide was even more potent in stimulating axon growth. Significant effects were observed at a concentration as low as 5 nM while other fatty acid ethanolmines including DPAn-6 ethanolamide (DPEAn-6), anandamide (AEA) and oleoylethanolamide (OEA) exerted no effects at 10 nM (Fig. 11).

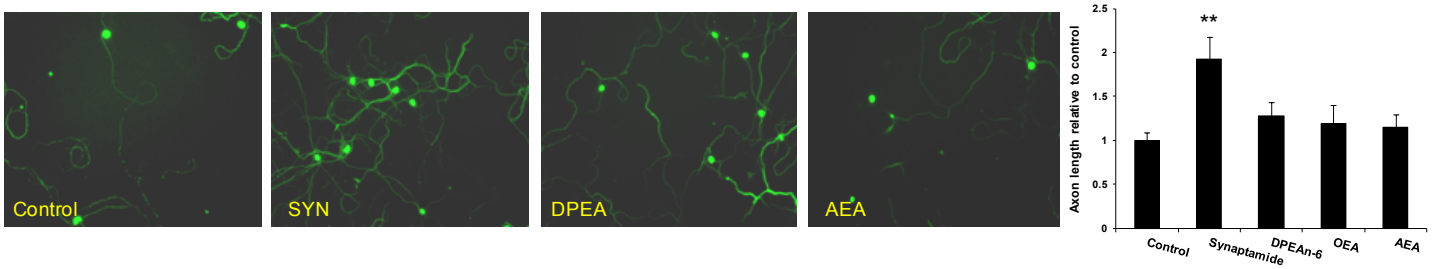


Fig. 11. Effect of polyunsaturated fatty acid ethanol amides on cortical axon growth. Cortical neuron cultures were treated with 10 nM fatty acid amides for 3 days, immunostained for axon specific marker SMI-312 and axon length was quantified by Metamorph software. **, $p < 0.01$ vs. control.

To monitor the effects of DHA and its metabolites on axon repair, we are in the process of establishing an *in vitro* axon injury model using a microfluid culture platform. In this model, cortical neurons are seeded in one side of a culture chamber and axons are allowed to grow through multiple grooves to reach the other side of the chamber (Fig. 12). At the end of the grooves (dotted line), axons are severed by aspiration of the media from the axonal compartment for 5 sec, and regrowth of axon is monitored using axon specific markers such as SMI-312. This device was also used to confirm the positive effect of synaptamide on axon growth (Fig. 13). Once the model is established, we will examine the effects of DHA or specific DHA metabolites on axon repair.

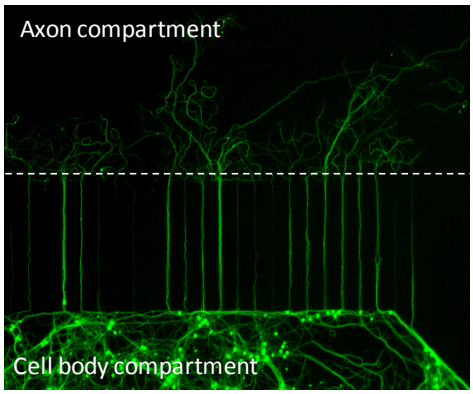


Fig. 12. Axon (SMI-312 positive, green) outgrowth in an axon device observed for cortical neurons after 14 days in vitro culture.

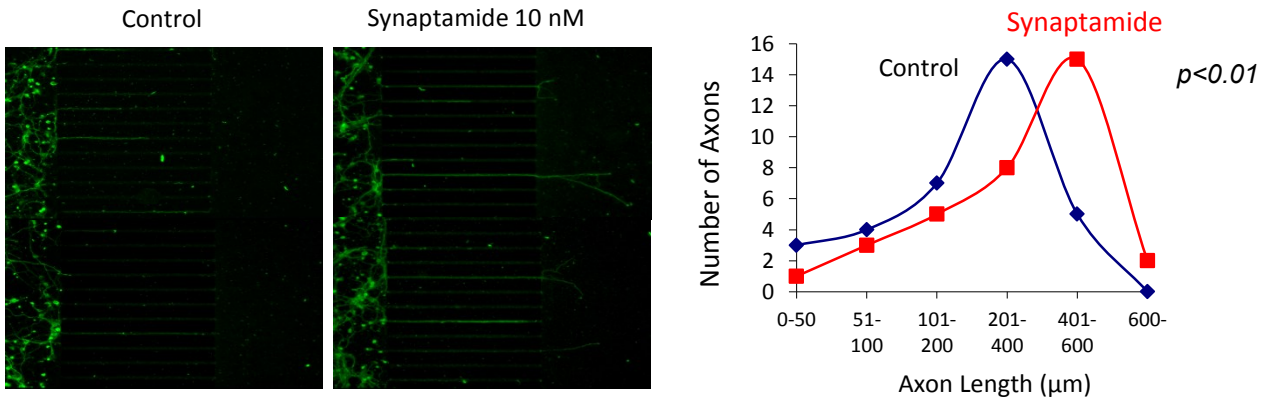


Fig. 13. Effect of synaptamide on axon outgrowth in an axon device after cortical neurons were cultured with 10 nM synaptamide for 72 h.

Task 3: Developing software in consultation with an instrumental company software team to establish a method to profile a broader range of DHA metabolites (months 6-18)

Formation of additional DHA metabolites in the cortical cultures was examined using the stable isotope labeling assisted metabolomics approach using the high resolution MS that has been recently installed. A new software "IsotpeLabelFinder" was developed in collaboration with Thermo Instrumental Company using the mass difference between natural and U-C13-labeled DHA metabolites and the distinct isotopic profile of the C13-labeled metabolites. Using this approach, we are in the process of identifying metabolites of DHA formed in the cortical neuron cultures and brain homogenates.

Year 2 Key Research Accomplishments

1. Dietary conditions to generate extreme and moderate DHA depletion in the mouse brain has been established for testing TBI outcome.
2. Using a mouse model of TBI, we established the adverse effects of both extreme and moderate DHA depletion on spontaneous recovery from injury.
3. Effect of DHA and synaptamide on axon growth has been established in cortical neuronal culture.
4. An axon growth model using a microfluid culture platform has been established for further development into an *in vitro* injury model.
5. An algorism for stable isotope assisted identification of DHA metabolites has been established.

Year 3

Task 1: Testing therapeutic potential of DHA and/or DHA metabolites administration on recovery after TBI (months 25-30)

During this report period, we evaluated the time course profile of DHA in blood in preparation of testing the therapeutic potential of DHA and to optimize the DHA administration protocol. Prior to that, we established an adverse impact of moderate DHA-depletion on the functional recovery since moderate depletion is rather commonly observed with the western dietary practice. In addition, we demonstrated deleterious effects of DHA-depletion in both genders.

During the last report period, we successfully generated mice with varying degrees of DHA depletion in the brain by feeding an omega-3 deficient special diet for one to three consecutive generations. We established that DHA-adequate mice recover significantly better from TBI compared to the DHA-deficient mice in the extreme case of DHA-depletion (over 70%). Histological and biochemical measures such as NeuN positive cells and spectrin alpha cleavage also consistently indicated that DHA-adequate mice recover better from TBI. Our findings from mice with extreme DHA-deficiency have been published in PLoS One during this period. To address the situation rather commonly observed with Western dietary practice, we extended our investigation to moderate DHA-depletion (by 30%). Although the extent was not as severe as the extreme case of DHA-depletion, both vestibulomotor functions assessed by rotarod and beam walk tests as well as memory evaluated by the novel object recognition test indicated significant differences between adequate and deficient groups with the latter showing slower recovery. In addition, cognitive function and anxious behavior were also adversely affected by the moderate omega-3 fatty acid deficiency in TBI-inflicted mice. Modest increases in the level of cleaved alpha spectrin II in the cortex of DHA-deficient injured mice as compared to the DHA-adequate controls also supported the adverse impact of moderate DHA-depletion.

Above observations were made in male mice. Even though females constitute of about half the total population, females are seldom used as experimental models because of the possibility that hormonal fluctuations may confound the experimental results. We moderately depleted the brain DHA content in female mice by diet and assessed the recovery after TBI. We found that there is a similar adverse impact of DHA depletion on recovery

in terms of motor deficits assessed by beam walk test (Fig. 1) and memory assessed by fear conditioning. These experiments imply that DHA depletion may have deleterious effects irrespective of gender.

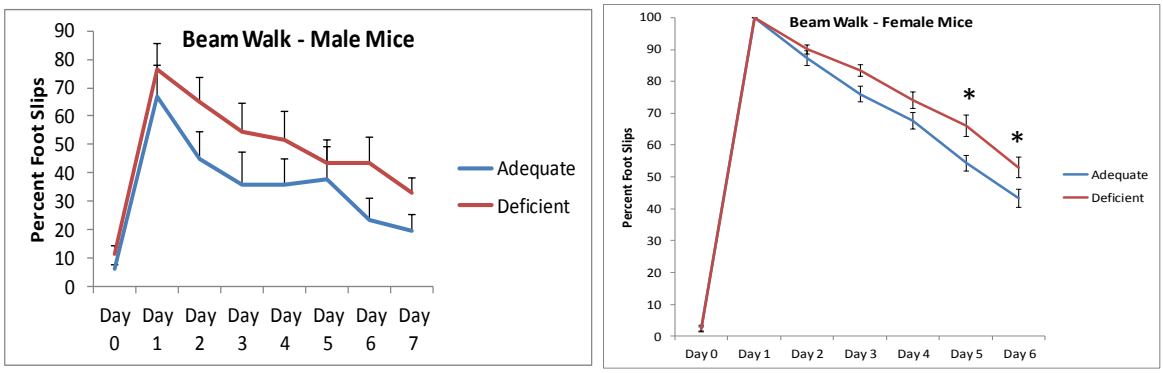


Fig. 1. Adverse impact of brain DHA depletion on the functional recovery after TBI irrespective of gender. *, $p<0.05$.

During this period, we started testing therapeutic potential of DHA and the DHA metabolite synaptamide. To ascertain the peak blood levels of DHA after oral or intraperitoneal (i.p.) administration, DHA was dissolved in 5% solutol plus 5% dimethylacetamide in saline or water (for i.p. or oral gavage at 25 mg/kg i.p. or 500 mg/kg, respectively). Blood was collected at 0, 1, 2 and 4 hours after DHA administration and lipids were extracted and analyzed. Both i.p. and oral administration produced similar serum DHA concentration profiles with peak at 1 h after administration (Fig. 2). Using this protocol, we are in the process of testing the effects of DHA administration on TBI outcome.

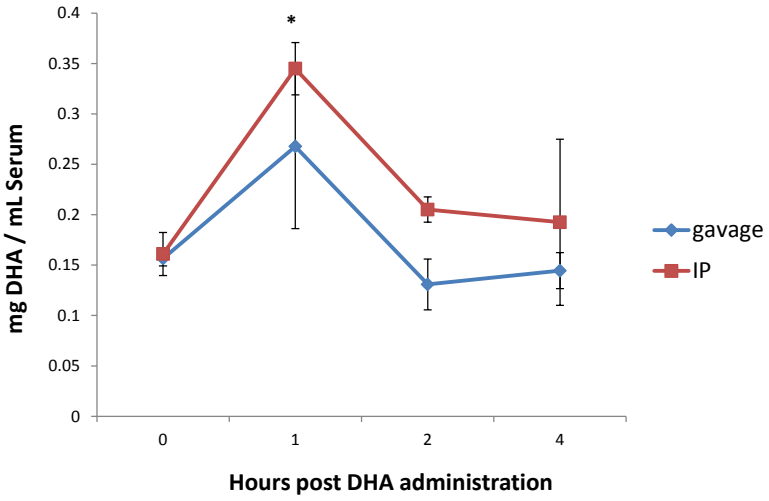


Fig. 2. Time course of the serum DHA level for gavage or i.p. administration of DHA. *, $p<0.05$ vs. 0 h control.

We have previously identified *N*-docosahexaenoylethanolamine (synaptamide) as a potent neuritogenic, synaptogenic and neuritogenic metabolite of DHA formed in hippocampal and cortical neuronal cultures. To test the effects of synaptamide on axon repair, we have also examined the regrowth of axons after injury during this review period. We first established an *in vitro* axon injury model using a microfluidic culture platform. In this model, cortical neurons were seeded in one side of a culture chamber and axons were allowed to grow through multiple grooves to reach the other side of the chamber. At the end of the grooves (dotted line), axons were severed by rapid aspiration of the media from the axonal compartment, and regrowth of axon was monitored using the axon specific marker SMI-312. This model allowed us to find that synaptamide at a concentration as low as 10 nM stimulated axon regrowth in axotomized cortical neurons (Fig. 3), indicating the potential of synaptamide for axonal repair after injury.

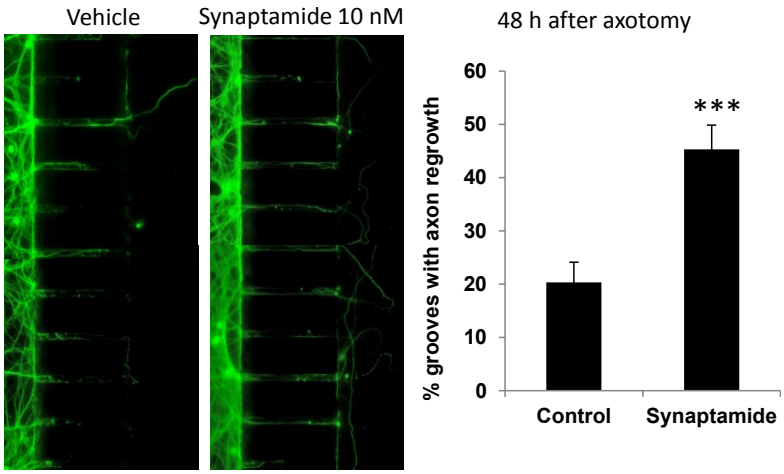


Fig. 3. Regrowth of axon (SMI-312 positive, green) evaluated in an axon device after 14 days *in vitro* culture of cortical neurons followed by axotomy and synaptamide treatment. ***, $p<0.001$

Task 2: Analyzing active metabolites in the control and posttraumatic brains during the course of recovery after dietary manipulation of DHA status or DHA administration (months 18-36)

Using the mass spectrometric analysis method that we established, we measured the TBI-induced metabolite formation time course during this period. We found that there are three distinctive classes of metabolites in terms of the peak production time. This information will enable us to optimize the DHA or metabolite injection time to improve TBI outcome.

The C57BL/6N male mice were injured by CCI delivered after craniotomy to the left hemisphere of the brain. The mice were euthanized at specific time intervals after TBI by cervical decapitation. The brain was rapidly removed and the cortex around the injury site and hippocampus from the injured hemisphere and the corresponding parts from the uninjured hemisphere were dissected, immediately frozen in a dry ice/isopropanol slurry and stored at -80° C until analysis. Tissues were homogenized and lipids extracted via reverse phase solid phase extraction in the presence of a mixture of deuterated internal standards. The extract was analyzed via HPLC-MS and HPLC-MS/MS in the negative ion mode. Metabolites were identified by MS/MS and with the help of corresponding internal standards when they are available. Levels of metabolites as a function of time after TBI were evaluated by comparison of peak areas with internal standards using either the [M-H]- or a unique fragment ion. Quantitation of the metabolites for which the internal standard of identical structure was not available was based on a relative term.

We found three distinctive patterns in the time course of the TBI-induced metabolite formation. Prior to injury, synaptamide and anandamide levels in the cortex were about 0.01 fmol/µg protein. At 1 h after TBI, synaptamide levels in the injured cortex increased to 0.3 fmol/µg protein. Anandamide also increased to about 0.3 fmol/µg protein in the same period. Both synaptamide and anandamide levels increased through 48 h post-injury to 8.9 and 3.0 fmol/µg protein, respectively (Fig. 4a). It has not been tested whether their levels would continue to rise past 48 hours. Although the total DHA and AA content was found to be about 15 and 10 mole %, respectively, the level of free AA is significantly greater than that of DHA at time points examined (Fig. 4b). For example, the AA and DHA levels increased from about 2 and 0.2 pmol/µg protein at the basal condition to 3.4 and 2.6 pmol/µg protein at 48 h after TBI, respectively. Nevertheless, the synaptamide level after TBI is significantly higher than anandamide, suggesting preferential synthesis of synaptamide after TBI.

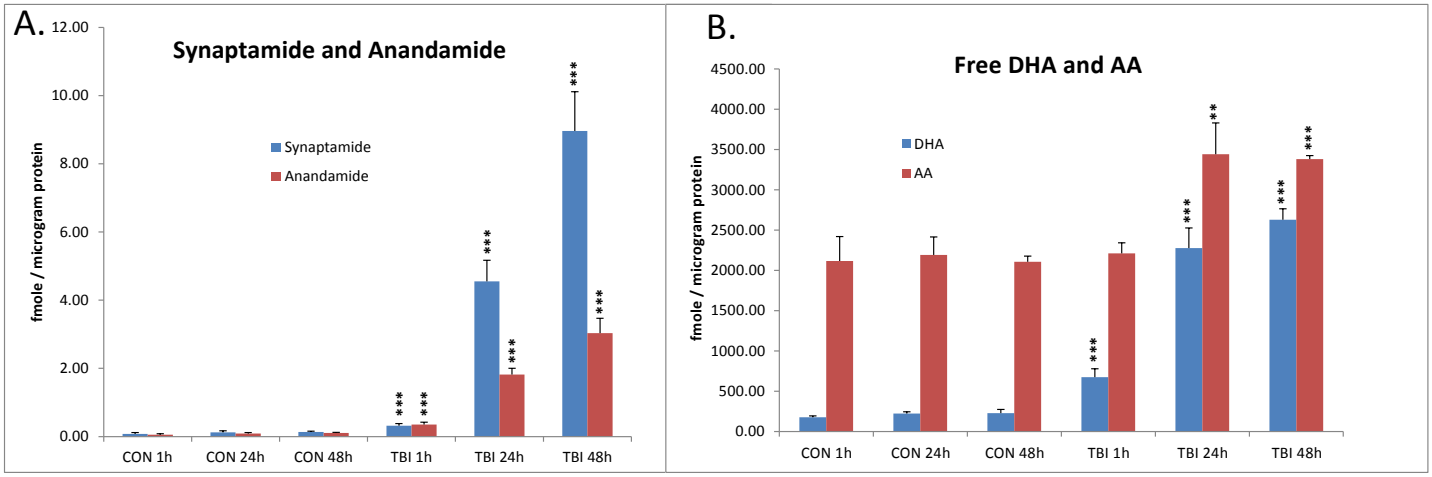


Fig. 4. Time course of TBI-induced formation of fatty acid ethanolamides (A) and free fatty acids (B). **, $p<0.01$; ***, $p<0.001$ vs. corresponding controls.

Cyclooxygenase metabolites of AA including thromboxane and prostaglandins showed a more acute time course. Thromboxane B2 (TXB2), PGF2-alpha and PGD2/E2 peaked at 1 h, followed by a downward trend at 3 and 6 h after TBI (data not shown) to about baseline by 24 h after injury (Fig. 5). Most monohydroxy derivatives of both DHA and AA (HDoHE and HETE) except 12- and 15-lipoxygenase products increased within 1 h after TBI, reached close to peak values by 24 h and remained highly elevated through 48 h (Fig. 6). The level of 12- and 15-lipoxygenase products 14- and 17-HDoHE as well as 12-HETE peaked at 24 h and decreased significantly by 48 h (Fig. 7). The indicated time course of specific metabolites will be the basis for selecting optimum DHA administration time points for testing therapeutic potential in the extended budget period. In addition, we are in the process of measuring the active metabolite status in DHA-adequate and deficient animals where significant differences in functional recovery outcome have been demonstrated.

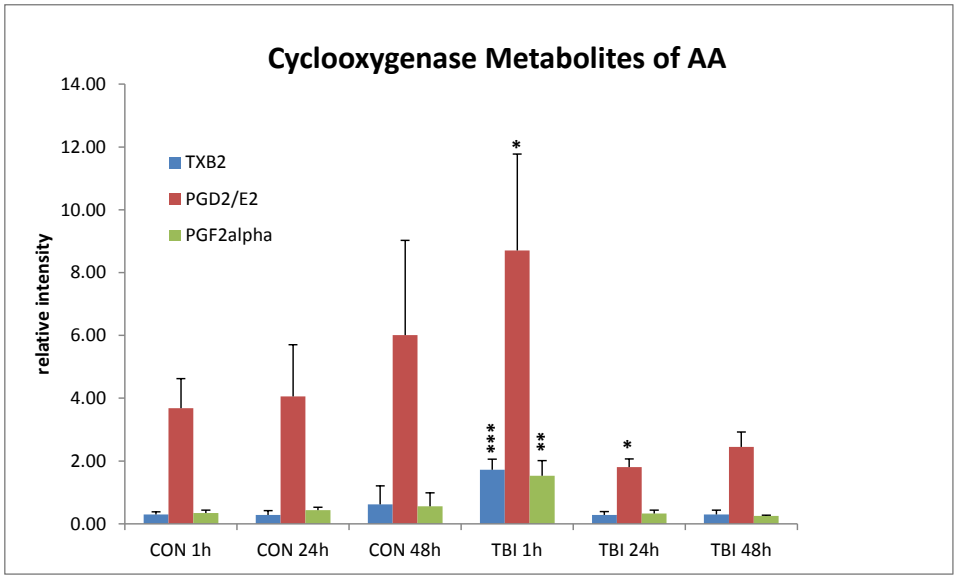


Fig. 5. Time course of TBI-induced formation of cyclooxygenase products of AA. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$ vs. corresponding controls.

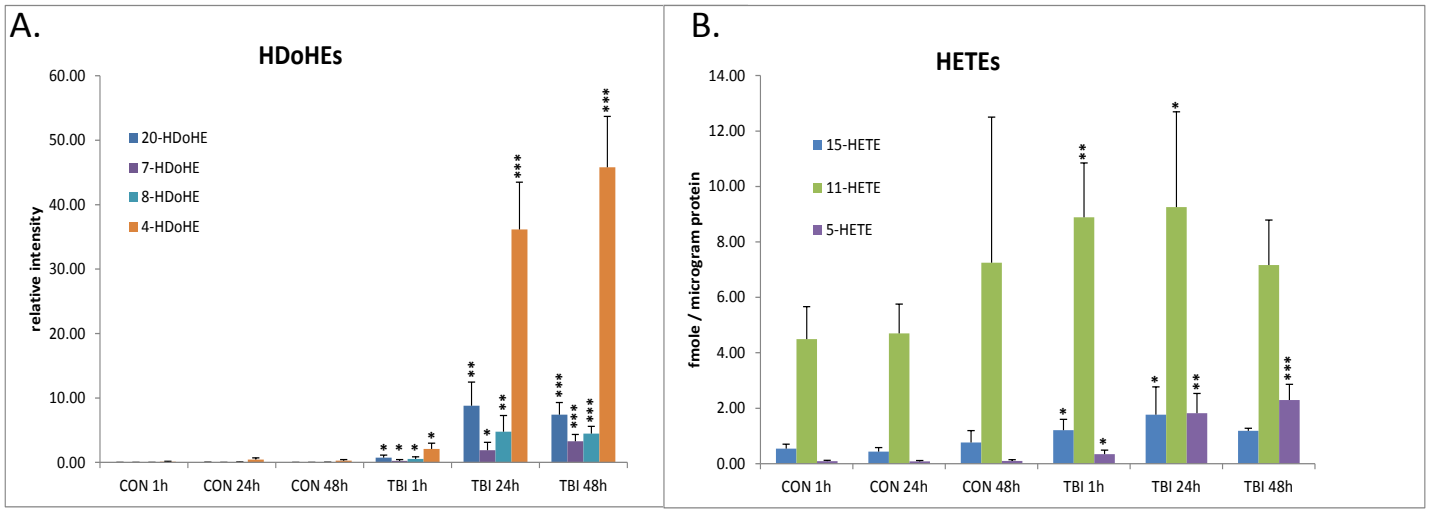


Fig. 6. Time course of TBI-induced formation of DHA- (A) and AA-derived (B) monohydroxy products. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$ vs. corresponding controls.

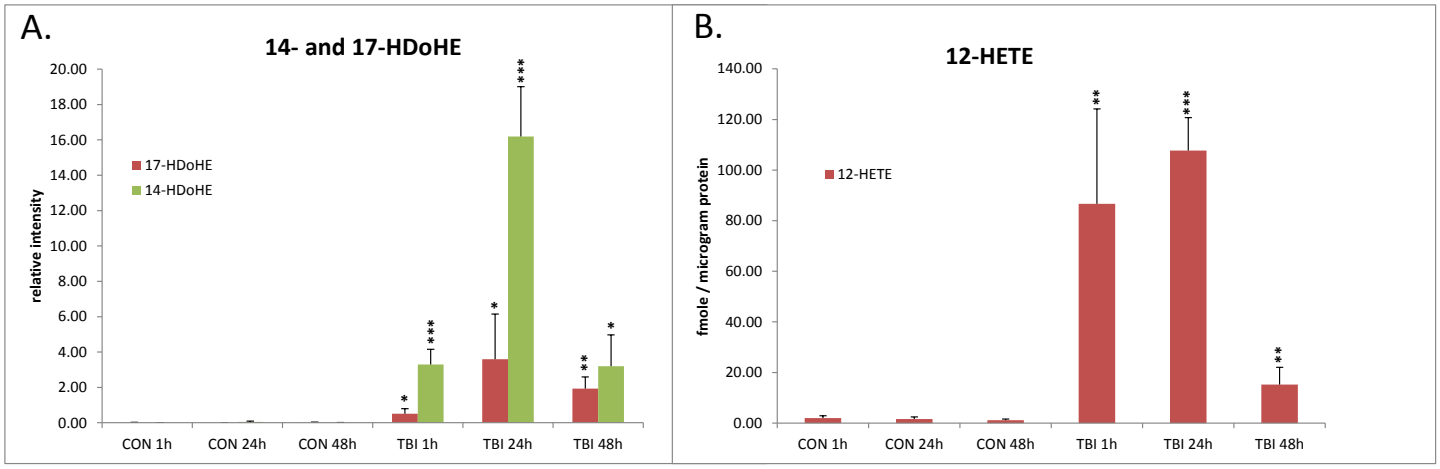


Fig. 7. Time course of TBI-induced formation of 12- and 15-lipoxygenase products of DHA (A) and AA (B). *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$ vs. corresponding controls.

Task 3: Optimizing the DHA administration protocol (months 31-36)

We are in the process of testing the effects of DHA administration on functional recovery. We are still optimizing the administration protocol in terms of administration rout, time and dose as well as diet selection after injury. We will accomplish this during the no cost extension period.

Year 3 Key Research Accomplishments

1. Using a mouse model of TBI, we established the adverse impact of moderate DHA-depletion on spontaneous recovery from injury.
2. We demonstrated the adverse effects of DHA-depletion in both genders.
3. Using an axon growth model using a microfluidic culture platform, an *in vitro* injury model was established. Preliminary results indicate-stimulated axon regrowth with synaptamide treatment
4. The time course of TBI-induced metabolite formation from DHA and AA was determined.

Year 4 (No cost extension period)

Task 1: Testing effects of DHA status on TBI outcome using injury and dietary models established

To establish the relationship between the brain DHA status and TBI outcome, we continued to examine the effect of varying extent of DHA-depletion on the functional recovery during this report period. Despite the less extent compared to the extreme case, the moderate DHA depletion which is commonly observed with the western dietary practice, also significantly worsened the recovery outcome. Proinflammatory cytokines were significantly upregulated and neuronal cell death was exacerbated in the TBI-inflicted moderately DHA-depleted brains.

During the last report periods, we successfully generated mice with varying degrees of DHA depletion in the brain by feeding an omega-3 deficient special diet for one to three consecutive generations. Previously, we have also established that extreme DHA depletion in the brain (brain DHA composition of 4%) significantly exacerbates functional recovery after TBI (Desai et al, 2014). To further establish the relationship between the brain DHA and TBI outcome, we examined spontaneous recovery after TBI in the group with moderate DHA-depletion (30-40% depletion) in comparison to the adequate group. The 10-12 weeks old male mice from Adequate (15% brain DHA) and moderately depleted (9% brain DHA comparable to human levels) groups were subjected to controlled cortical impact (CCI)-induced TBI in the parietal cortex and the recovery outcome was evaluated by rotarod, beam walk and open field tests (Fig. 1). The mice with the 9% brain DHA level performed significantly worse, indicating the possibility to improve the outcome in humans with DHA supplementation after injury. The extent of DHA enrichment has significant impact on the recovery outcome as indicated by the beam walk performance in Figure 2 for mice with three different brain DHA levels (Fig. 2). These data suggest that individual differences in the brain DHA status may be a significant factor contributing to individual recovery outcome.

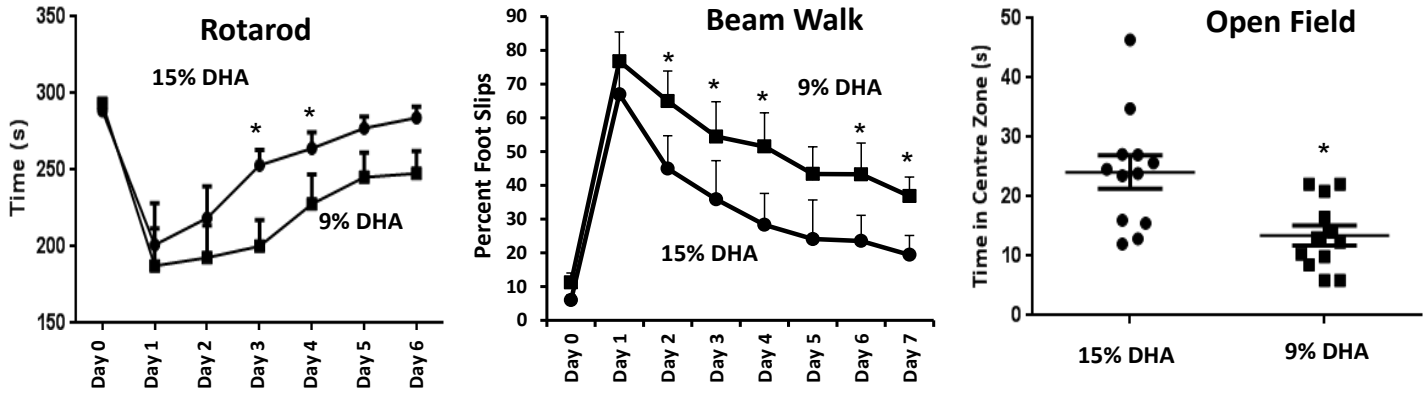


Fig. 1. Recovery outcome after CCI-induced TBI in mice with humanized brain DHA level (9%) and DHA adequate mice (15%). *, $p<0.05$

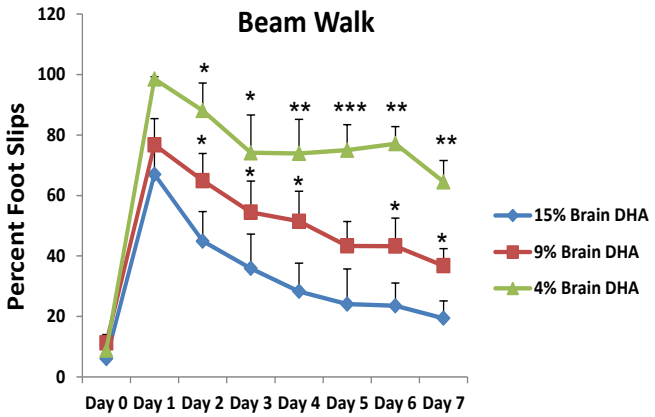


Fig. 2. TBI outcome evaluated by the beam walk test in mice with three different brain DHA levels. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$ vs. 15% DHA group.

Expression of pro-inflammatory cytokines such as TNF-α, IL-β, IL-6 and CCL2 was dramatically increased in the cortex around the injury site at 4 hours after TBI. This increase was further exacerbated in the moderately DHA-depleted mice, indicating that the n-3 fatty acid deficiency primes the brain to mount increased inflammatory response to injury (Fig. 3). An increase in the TUNEL positive cells was also observed in the cortex 3 days after TBI. This increase seemed to be greater in n-3 fatty acid deficient tissue, suggesting more cell death (Fig. 4).

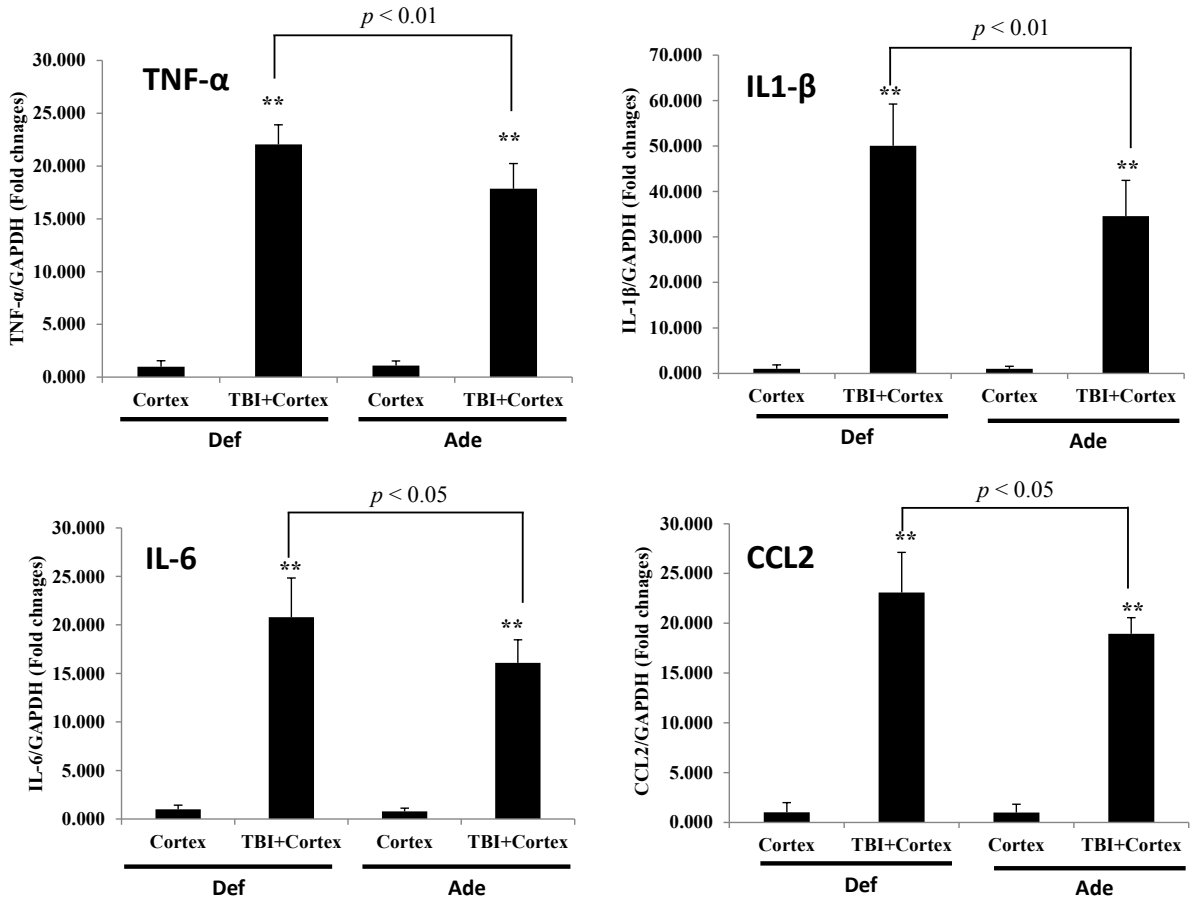
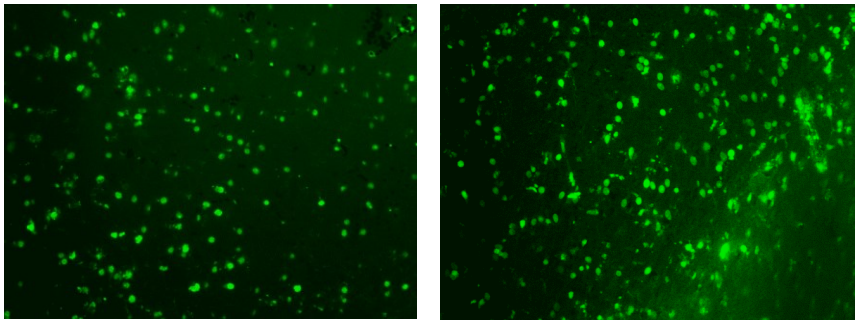


Fig. 3. Proinflammatory cytokine expression in the cortex from n-3 fatty acid adequate and deficient mice at 4 h after TBI. **, $p<0.01$



DHA-adequate (15% DHA) DHA-depleted (9% DHA)

Fig. 4. TUNEL staining to identify apoptotic cells in the cortex from n-3 fatty acid adequate and deficient mice at 3 days after TBI.

These studies are soon to be finalized for publication. As injury-induced microglial activation is a prominent source of pro-inflammatory cytokines, microglial profiles are being examined at different time points to discover differences due to the n-3 fatty acid status along with microglia phagocytic activity.

Task 2: Measuring the active metabolite status in DHA-adequate and deficient animals where significant differences in functional recovery outcome have been demonstrated.

Using the metabolite profiling method and parameters established during the last review period, we have measured active metabolites in TBI-inflicted animal brains. We found that TBI-induced formation of DHA metabolites was significantly affected by the brain DHA status.

Under our experimental conditions, the level of cyclooxygenase products of AA, TXB2, PGE2 and PGF2α, which peaked at 1h after TBI was not significantly altered by DHA depletion. Similarly, DHA-depletion in the brain did not affect the TBI-induced production of other AA metabolites such as HETEs. In contrast, the DHA metabolites, HDoHEs (Fig. 5) and synaptamide (Fig. 6), were significantly reduced in DHA-depleted mouse brains. Like other AA metabolites, the anadamide level was not altered, either. Since significant differences in functional recovery outcome have been demonstrated between DHA-adequate and deficient animals, these data suggested that TBI-induced production of DHA metabolites may exert protective effects and improve the recovery from TBI.

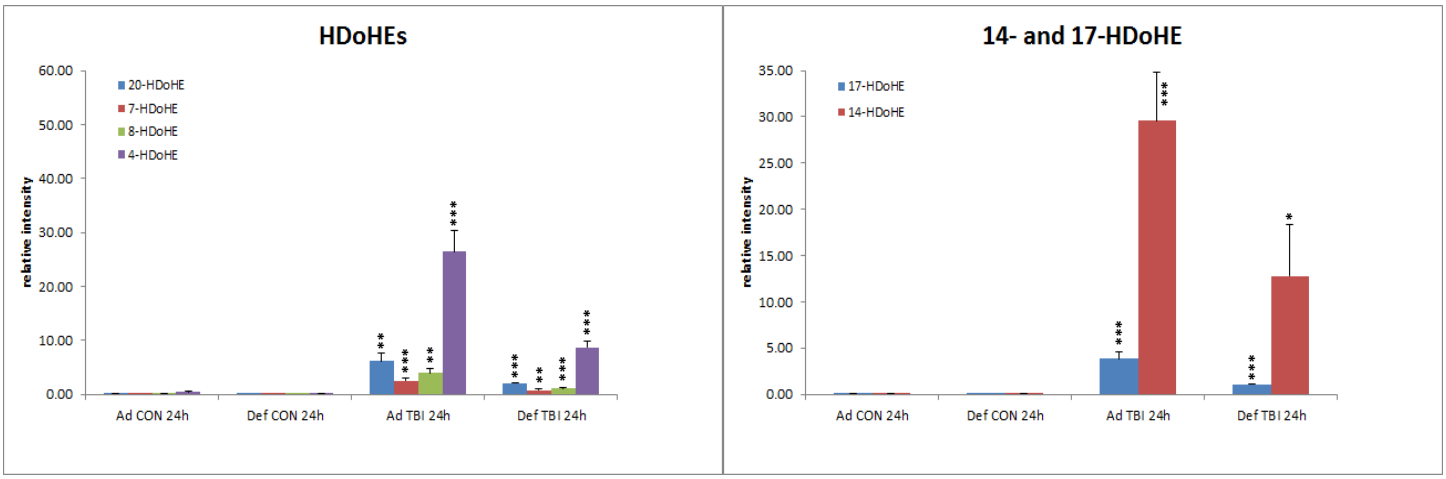


Fig. 5. Effect of the brain DHA status on the TBI-induced production of hydroxy docosahexaenoic acids (HDoHEs). *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$ vs. corresponding controls.

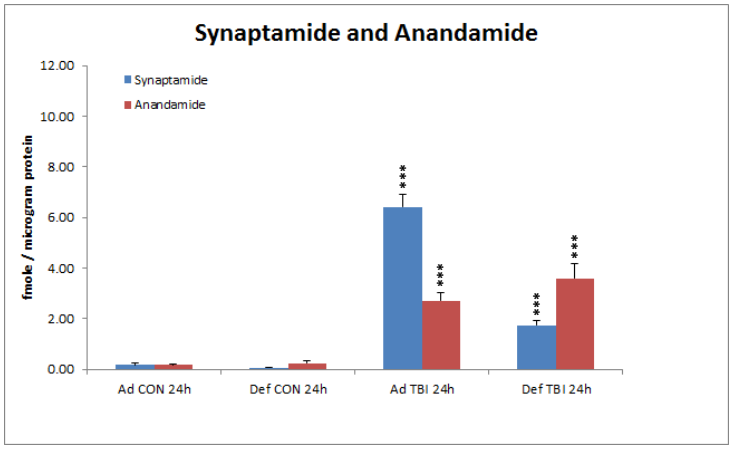


Fig. 6. Effect of the brain DHA status on the TBI-induced production of hydroxy docosahexaenoic acids (HDoHEs). ***, $p<0.001$ vs. corresponding controls.

Task 3: Testing therapeutic potential of DHA and/or DHA metabolites administration on recovery after TBI and optimizing the DHA administration protocol

During the no cost extension period, we tested the effects of DHA administration on functional recovery. We found that exogenous treatment of DHA did lead to improved recovery in some cases in learning and memory, suggesting therapeutic potential. Nevertheless, to improve reproducibility and to find optimum effects of DHA treatmenr, dose, dosage and rout of administration should be further optimized in future.

We tested whether post injury DHA supplementation can intervene with the propagation of the injury and improve the injury outcome. Experiments were conducted with DHA treatment at different dosages. The initial dose was selected as a relatively high dose to ensure that adequate levels of DHA are available after injury. For oral administration, free DHA was added to 1% bovine serum albumin in saline to increase its absorption after oral gavage. For intraperitoeal administration, DHA was dissolved in a solution of 5% solutol and 5% dimethylacetamide (DMAC) in saline. DHA was administered orally at a dose of 500 mg/kg either 2 hours before (pre-treatment) or 20 minutes after surgery (post-treatment). The DHA treated groups did not differ from the vehicle treated groups in the rotarod test indicating that motor recovery was not affected by the DHA treatment. However, the novel object recognotion test showed that the pre-treatment group explored the novel onject significantly more than chance (50%), indicating preserved cognition due to DHA pre-treatment (Fig. 7). Similar results were found for the post-treatment group as well, though this may have been due to low inter-subject variability within the group. The comparision between groups did not show significant effects of the treatment.

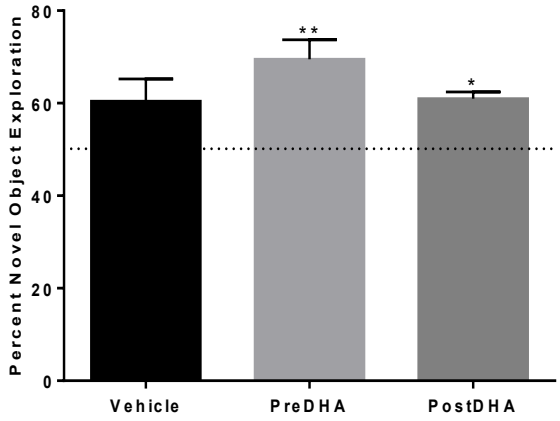


Fig. 7. Effect of single oral gavage of DHA at 500 mg/kg at 2 h before (preDHA) or 20 min after TBI (postDHA) on Novel object recognition test. *, $p<0.05$; **, $p<0.01$

This experiment was repeated using the same dose with two oral administrations of DHA for the post-treatment at 20 min and 24 h after injury. As before, there was no change in the motor deficits and general activity due to treatment. There was a trend to improve exploration in the novel object recognition test for both pre- and post-TBI DHA treatment, however, neither reached the statistical significance (Fig. 8). This may be due to the low number of animals in each group (n=5) in this test, which was due the elimination of some of the mice from each group due to lack of exploration.

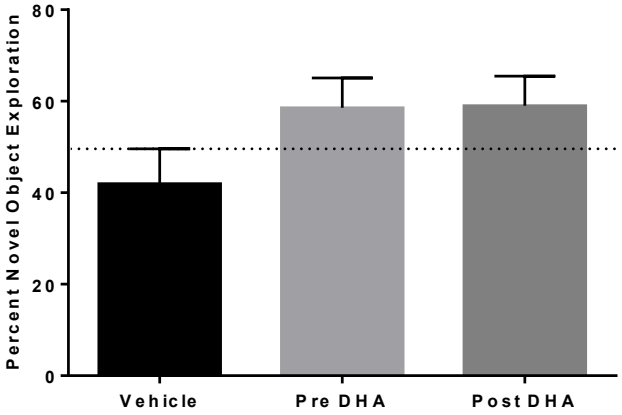


Fig. 8. Effect of single oral gavage of DHA at 500 mg/kg at 2 h before (preDHA) or two administrations at 2 and 24 h after TBI (postDHA) on Novel object recognition test.

In order to ascertain whether injecting DHA leads to better recovery, 100 mg/kg of DHA was injected intraperitoneally at 20 min, 1 day and 2 days after TBI. The treated group showed better performance in the novel object recognition test as compared to vehicle (Fig. 9), howevr, there was 25 percent mortality (2 out of 8) accompanied with poor performance in the rotarod test.

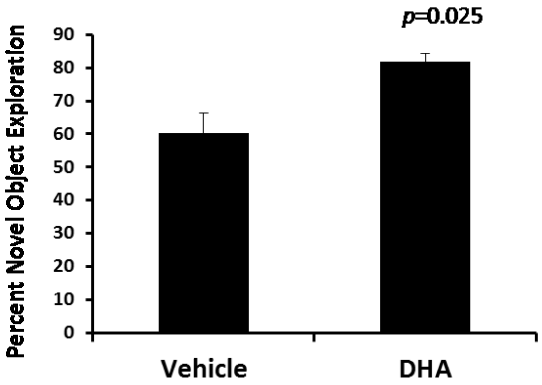


Fig. 9. Effect of three times of DHA injections (i.p.) at 100 mg/kg at 20 min, 1day and 2 days after TBI on Novel object recognition test.

In summary, the exogenous treatment of DHA did lead to improved recovery in some cases in learning and memory, suggesting therapeutic potential. Nevertheless, to improve reproducibility and to find optimum effects of DHA treatmenr, dose, dosage and rout of administration should be further optimized in future. DHA is a polyunsaturated fatty acid that is prone to being rapidly oxidised and metabolized. We therefore concentrated on using higher doses and have not experimented with lower doses. Also, we have limited the routes of administration to oral and intraperitoneal and have not yet tested intravenous and central injections.

Year 4 Key Research Accomplishments

1. Using a mouse model of TBI, we established the adverse impact of moderate DHA-depletion on spontaneous recovery from injury.
2. We demonstrated the reduction of DHA-metabolites produced after TBI.
3. We demonstrated upregulation of proinflammatory cytokines in TBI-inflicted, moderately DHA-deficient mouse brains.
4. The therapeutic potential of DHA administration on recovery after TBI was tested, and the optimization of the DHA administration protocol was attempted.

Reportable Outcomes (during the entire award period)

Presentations:

1. Desai A, Kevala K and Kim HY. The Impact of Dietary Omega-3 Fatty Acid Deficiency on The Outcome of Traumatic Brain Injury. NIH Research Festival 2012, Bethesda, MD
2. Kharebava G and Kim HY. Regulation of Axonal Morphogenesis by Docosahexaenopic Acid and Its Ethanolamide Derivative, Synaptamide. NIH Research Festival 2012, Bethesda, MD
3. Kharebava G and Kim HY. Axonal Morphogenesis Affected by Docosahexaenoic Acid and Its Ethanolamide Derivative, Synaptamide. Society for Neuroscience meeting 2012, New Orleans, LA
4. Desai A, Kevala K and Kim HY. Omega-3 Fatty Acid Deficient Diet Worsens Traumatic Brain Injury Outcome. Society for Neuroscience meeting 2012, New Orleans, LA (selected for nanosymposium presentation)
5. Kevala, K., Rashid, M.D., Sanders, M., Kim, H.Y. Identification of Novel Metabolites of Docosahexaenoic Acid in Neural Stem Cells Using Stable Isotope Labeled Compounds and High-Resolution Mass Spectrometry, 61st ASMS Conference on Mass Spectrometry, 2013, Minneapolis, MN
6. Kim HY. Omega-3 Fatty Acid-Derived Neuroprotective Mechanisms in Traumatic Brain Injury. 2014, National Capital Area TBI Research Symposium, Bethesda, MD
7. Kevala, K., Desai A., Kim, H.Y. Analysis of Docosahexaenoic and Arachidonic Acid Derived Metabolites in Brain After Traumatic Brain Injury by High Resolution LC-MS/MS, 62nd ASMS Conference on Mass Spectrometry, 2014, Baltimore, MD
8. Desai A, Barnes J, Kevala K and Kim HY. Behavioral Changes in DHA Deficient Female Mice after Traumatic Brain Injury. Society for Neuroscience meeting, 2014, Washington DC
9. Barnes J, Desai, Kevala K, A Rashid M and Kim HY. Moderate Docosahexaenoic Acid Deficiency Impairs Recovery from Traumatic Brain Injury, Society for Neuroscience meeting, 2014, Washington DC
10. Kharebava G, Sarkar S and Kim HY. Role of PP2A in N-Docosahexaenoylethanolamine Mediated Axonal Development. 2014 Gordon Research Conference, Cell Biology of the Neuron, Waterville Valley, NH
11. Desai A, Kevala K and Kim HY. The Impact of Dietary Omega-3 Fatty Acid Deficiency on the Outcome of Traumatic Brain Injury. NIH-KSA 2015, Bethesda, MD
12. Desai A, Park T, Kevala K and Kim HY. n-3 Polyunsaturated Fatty Acid Deficiency Impairs Recovery from Traumatic Brain Injury. NIAAA Poster 2015, Rockville, MD

Publications:

1. Desai A, Kevala K, Kim HY Depletion of brain docosahexaenoic acid impairs recovery from traumatic brain injury. *PLoS One* 9:e86472, 2014.
2. Kim HY. Neuroprotection by Docosahexaenoic Acid in Brain Injury. *Mil Med*. 179:106-111, 2015.

Conclusion (for studies during the entire award period)

During the ward period, we have completed the study on effect of the DHA status on TBI outcome and published a paper in PLoS One. We have established an animal model of TBI and dietary models for generating varying degrees of brain DHA depletion. Using these models, we demonstrated that DHA-adequate brains are more resilient to injury. Spontaneous functional recovery after TBI, motor and cognitive function, was significantly better in DHA-adequate mice, compared to both moderately (<35%) or severely DHA-depleted (>70%) animals. An adverse impact of DHA-deficiency on functional recovery from TBI was observed

regardless of the gender. We also established a novel metabolite search method using stable isotopes and mass spectrometry, and now a manuscript is being prepared for communication. Using quantitative mass spectrometric approaches we identified increased formation of bioactive DHA and AA metabolites after TBI. We demonstrated positive bioactivity of DHA-metabolites, particularly synaptamide, on axon growth using cortical neuron cultures, and a manuscript is in preparation from this study. We established an axon injury model by which bioactivity of synaptamide was further extended to axon regeneration. We initiated testing the therapeutic potential of DHA and synaptamide in TBI, and attempted to optimize dose and time windows of the treatment using FAAH KO and wild type animals. Preliminary data indicated positive effects of DHA and synaptamide on functional recovery from TBI, but further research is required for the optimization of the administration protocol.

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PLOS ONE

Depletion of Brain Docosahexaenoic Acid Impairs Recovery from Traumatic Brain Injury

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Abstract

Omega-3 fatty acids are crucial for proper development and function of the brain where docosahexaenoic acid (DHA), the primary omega-3 fatty acid in the brain, is retained avidly by the neuronal membranes. We investigated the effect of DHA depletion in the brain on the outcome of traumatic brain injury (TBI). Pregnant mice were put on an omega-3 fatty acid adequate or deficient diet from gestation day 14 and the pups were raised on the respective diets. Continuation of this dietary regime for three generations resulted in approximately 70% loss of DHA in the brain. Controlled cortical impact was delivered to both groups of mice to produce severe TBI and the functional recovery was compared. Compared to the omega-3 adequate mice, the DHA depleted mice exhibited significantly slower recovery from motor deficits evaluated by the rotarod and the beam walk tests. Furthermore, the DHA deficient mice showed greater anxiety-like behavior tested in the open field test as well as cognitive deficits evaluated by the novel object recognition test. The level of alpha spectrin II breakdown products, the markers of TBI, was significantly elevated in the deficient mouse cortices, indicating that the injury is greater in the deficient brains. This observation was further supported by the reduction of NeuN positive cells around the site of injury in the deficient mice, indicating exacerbated neuronal death after injury. These results suggest an important influence of the brain DHA status on TBI outcome.

Citation: Desai A, Kevala K, Kim H-Y (2014) Depletion of Brain Docosahexaenoic Acid Impairs Recovery from Traumatic Brain Injury. PLoS ONE 9(1): e86472. doi:10.1371/journal.pone.0086472

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Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Traumatic brain injury (TBI) is a leading cause of death and disability contributing to a third of all injury-related deaths in the USA [1]. Although people engaged in certain professions such as military personnel, amateur and professional athletes are at a higher risk of suffering from TBI, it is encountered in all populations. TBI can manifest across a wide range of responses, depending on the severity of injury. Mild concussion can lead to temporary state of confusion and transient unconsciousness while severe brain injury may result in loss of function of the limbs, speech impairment, disturbances in normal memory and emotional responses. Excitotoxicity, oxidative stress and inflammation are the primary mechanisms that lead to neuronal cell death and dysfunction in models of brain injury [2].

The polyunsaturated fatty acids linoleic (LA, 18:2n-6) and linolenic acid (LNA, 18:3n-3) are essential fatty acids that cannot be synthesized by the body. LNA serves as the precursor for long chain omega-3 fatty acids such as docosahexaenoic acid (DHA) while LA is converted into long chain omega-6 fatty acids such as arachidonic acid (AA) [3]. DHA and AA are abundantly found in the brain, where these are stored mainly in membrane phospholipids. Immediately after the injury, phospholipases are activated, leading to an increase in free fatty acids [4]. DHA has been shown to increase neurite outgrowth and synaptogenesis, and promotes glutamatergic neurotransmission through increase in glutamate receptor subunit expression [5]. DHA can directly interact with nuclear receptors such as retinoid acid X receptor, activating transcriptional activity [6]. Moreover, DHA has been shown to be converted to anti-inflammatory, proresolving and neuroprotective mediators, such as resolvins [7] and protectins [8]. It has been also demonstrated that DHA metabolizes to synaptamide that has neuritogenic/synaptogenic properties [9]. In contrast, AA is converted by cyclooxygenases into 2-series prostaglandins and 4-series leukotrienes, most of which exert pro-inflammatory effects [10]. Supplementation of DHA exerts neuroprotective effects and has been reported to afford protection from diffuse axonal injury [11] and mixed brain injury [12] as well as in Alzheimer's disease model [13], cerebral ischemia [14,15], and Parkinson's disease [16,17]. However, not much is known regarding the effect of pre-existing membrane levels of DHA on the outcome after cortical impact injury. In the present study, we have investigated the consequence of severe depletion of brain DHA on the behavioral and histological outcome of focal brain injury in a mouse model after manipulating the brain DHA status through multi-generational feeding with an omega-3 fatty acid deficient diet.

Materials and Methods

Animals and Diets

Pregnant E14 C57BL/6J mice procured from the Jackson Laboratory were placed on either omega-3 fatty acid adequate or deficient diet as described earlier [5]. The modified AIN-93G [18] diets with custom fat mixture were procured from Dyets Inc.

(Bethlehem, PA, USA). The lipids in the omega-3 adequate diet consisted of tocopherol-stripped safflower oil (17.7 gm/kg), flaxseed oil (4.81 gm/kg), hydrogenated coconut oil (74.49 gm/kg) and alga-derived DHA oil (DHASCO; DSM-Martek Biosciences, Columbia, MD, USA) (3 gm/kg). The lipids in the omega-3 fatty acid deficient diet were tocopherol-stripped safflower oil (19 gm/kg) and hydrogenated coconut oil (81 gm/kg) (Table 1). The resulting fatty acid composition (%) of adequate vs. deficient diets was as following; LA, 13.8 vs. 13.8; LNA, 2.5 vs. 0.09; DHA, 0.9 vs. 0; and no AA or eicosapentaenoic acid (EPA) was detected. The litters were weaned on the same diet as their dams and considered the second generation on the adequate or deficient diet. They were mated around 3 months of age to produce the third generation pups that were again placed on the same diet after weaning as their dams. The nervous tissue of the third generation mice was substantially depleted of DHA. Mice from the third generation were used for the TBI experiments when they were 3-4 months old.

TBI model

All experimental procedures were approved by the institutional Animal Care and Use Committee (LMS-HK-03) and the US Army Medical Research and Materiel Command (USAMRMC) Animal Care and Use Review Office (ACURO), and performed according to the NIH Guide for Care and Use of Laboratory Animals. TBI was inflicted by controlled cortical impact using the Head Impactor TBI 0310 (Precision Systems and Instrumentation, LLC), a pneumatically contolled impactor for precise injury. Each mouse was anaesthetized using 4% isofluorane and fixed onto a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA). The anesthesia was maintained with 2.5% isoflurane. A midline incision was made on the scalp and a 4 mm craniotomy was made over the left cerebral hemisphere between the lambda and the bregma about 1 mm from the midline. The stereotaxic frame was inclined so as to make the plane of the cortex perpendicular to the impactor tip. The impact velocity was set at 3.5 m/s with the penetration depth at 1.5 mm and the impactor dwell time was 500 ms. After the injury, the wound was covered with Surgicel (Johnson & Johnson, Arlington, TX, USA) and the craniotomy was closed using cyanoacrylate. The body temperature of the mouse was maintained around 36–37°C with an infra-red warmer throughout the surgical procedure. The operated mice were placed in cages over a warm water blanket after surgery and allowed to recover with free access to the same diet as before surgery. Sham operated mice received craniotomy without brain injury.

Table 1. Composition of omega-3 fatty acid adequate and omega-3 fatty acid deficient diets[1].

	Adequate Diet		Deficient Diet	
	gm/kg	*kcal/gm*	*gm/kg*	*kcal/gm*
Vitamin Free Casein	200	744	200	744
L-Cystine	3	12	3	12
Cornstarch	150	540	150	540
Sucrose	100	400	100	400
Dextrose	199.5	726	199.5	726
Maltose Dextrin	150	570	150	570
Cellulose	50	0	50	0
Tocopherol Stripped Safflower Oil	17.7	159.3	19	171
Flaxseed Oil	4.8	43.3	0	0
Hydrogenated Coconut Oil	74.5	670.4	81	729
DHASCO[2]	3	0	0	0
Mineral Mix#210025	35	30.8	35	30.8
Vitamin Mix#310025	10	38.7	10	38.7
tBHQ[3]	0.02	0	0.02	0
Choline Bitartrate	2.5	0	2.5	0

1 These diets were prepared by Dyets Inc. (Bethlehem, PA, USA) based on AIN-93G (18) and have been used to achieve DHA depletion in rodents (24,36).
2 DHASCO: DHA Algal Oil.
3 tBHQ: tert-butylhydroquinone.
doi:10.1371/journal.pone.0086472.t001

Behavioral tests

Rotarod test. The rotarod test has been reported to be effective for evaluating motor deficits after TBI [19]. Rotarod apparatus from Med Associates (St. Albans, Vermont, USA) was used to assess the recovery in motor functions. Mice were trained on the accelerating rotarod for three days and scored on the fourth day, the day preceding the day of surgery. This score was considered the baseline Day 0 score. After surgery, mice were tested daily for six days. Each test session consisted of three trials separated by an interval of about ten minutes. The rotarod accelerated from 4 to 400 rotations per minute over the trial span of 5 min. The latency of mice to fall from the rotarod was recorded for each trial. The trial was also stopped after two consecutive passive rotations on the rotarod. The latency scores obtained from each trial were averaged.

Beam walk test. The beam walk test has been employed to assess fine motor coordination in rodents by counting the foot slips that occur while walking from one end of a narrow beam to the other [20]. A 50 cm long and 7 mm wide elevated plastic beam was used for this test. One training or test session was performed daily except on the day of surgery. Each daily session consisted of three trials, in which the number of hind-limb foot slips and the total number of steps were counted. The percent foot slips was calculated after adding the foot slips made and the steps taken in the three trials. Mice were trained to traverse the beam for two days and pre-surgery baseline performance was assessed on the third day. Daily assessments were made following the day of surgery.

Open field and novel object recognition tests. The novel object recognition test was used to assess the ability of mice to recognize and discriminate novel objects, which was taken as a measure of the memory of the familiar object [21]. From the fifth to the seventh day after TBI, the mice were allowed to explore an arena enclosed by a 40×4 cm open box made of black plexiglass (Stoelting, Wood Dale, Illinois, USA) for 5 min each day. The exploration of the arena for 5 min on the fifth day after surgery constituted the open field test. Following habituation on the seventh day, the mice were introduced into the arena which now had two identical objects. Each mouse was allowed to explore the two objects in the training period and the duration of exploration was monitored. The mouse was considered to explore an object if its nose was within 2 cm of the object and the mouse was sniffing or apparently focusing its attention on the object. This trial period was continued till the total object exploration time reached 30 seconds or for 15 min in the enclosure. One of the objects was replaced by a distinctly different object of comparable dimensions and the mice were individually re-introduced into the enclosure

three hours after the trial. The time of exploration of the familiar and the novel object was monitored for 4 min.

Fatty Acid Analysis

The tissues were homogenized in an ice cold mixture of 50/50 methanol/BHT (50 mg/L butylated hydroxytoluene) and buffer (15 mM tris, pH 7.4; 150 mM NaCl) with a Potter-Elvehjem homogenizer. Lipids were extracted by the method of Bligh and Dyer [22]. In brief CHCl3 and water were added to bring the $CHCl_3$:CH_3OH:H_2O ratio in samples to 2:2:1.8, followed by vigorous vortexing under nitrogen. Samples were spun at 3,000 rpm at 4°C and the bottom organic layers transferred to clean glass tubes. The extraction was repeated twice with the remaining aqueous layer. Pooled organic fractions were dried under nitrogen and suspended in 2:1 $CHCl_3$:CH_3OH. Fatty acids in the lipid extract were quantified by gas chromatography analysis after transmethylation with boron trifluoride/CH_3OH, followed by hexane extraction as described previously [23].

Western Blot Analysis

Brain cortical tissue of mice on omega-3 adequate and deficient diet groups was excised at 24 h and 7 days after TBI in two separate experiments. The α-spectrin cleavage was assessed at 24 h while synapsin 1 expression was assessed at 7 days after TBI. The tissue was homogenized manually in 2X lysis buffer (Cell Signaling Technology, Danvers, MA, USA) using a Potter-Elvehjem homogenizer, briefly sonicated and centrifuged to obtain the supernatant. Protein content was estimated and cortical samples were run in gradient polyacrylamide gels (Novex, Invitrogen, Carlsbad, CA, USA) and subsequently transferred onto a PVDF membrane (Millipore, Billerica, MA, USA). After blocking with 5% milk, mouse α-spectrin antibody (Santa Cruz Biotechnology, 1:800) or synapsin-1 antibody (Sigma, 1:1000) was used to probe for the respective protein, which were detected using Pierce supersignal west pico chemiluminescent detection substrate (Thermo Fisher Scientific, Rockford, IL, USA).

NeuN Immunostaining

50 μm free floating brain sections were cut with a vibratome. Five alternate brain sections from each mouse from the epicenter of injury were used for NeuN staining. All the staining steps were performed on a rocker. The sections were washed with PBS and permeabilized with 0.2% Triton X in PBS for 2 h. They were then immersed in 1% bovine serum albumin (BSA) in 0.1% tween 20 in PBS for 1 h. The sections were then immersed in anti-NeuN mouse monoclonal antibody (Millipore, 1:300) for 24 h on a rocker at 4°C. The sections were then washed with 0.1% tween 20-PBS thrice, immersed in Alexa-Fluor anti-mouse 555 (1:200) for 24 h in the refrigerator, washed thrice and mounted with immunocruz mounting medium (Santa Cruz Biotechnology, Inc.). The NeuN positive cells were observed using a 20X objective in 800 μm peri-contusion area. The images were captured using an inverted motorized IX81 Olympus microscope. For each non-overlapping field, a series of ten images was captured along the Z axis at 2 μm intervals and NeuN positive cells were counted for six fields per section using Metamorph software.

TBI lesion volume

For each brain, seven brain sections that were 400 μm apart and included the TBI lesion were mounted onto glass slides and stained with cresyl violet. The digital photograph of each 50 μm stained section was analyzed using ImageJ software (NIH, Bethesda, MD USA). The boundary of the lesion was carefully traced using the polygon tool and the lesion area was calculated. The lesion volume was then obtained by multiplying the sum of the lesioned area obtained from each section by the distance between the sections.

Statistical analysis

Two-way AVOVA was used followed by Holm-Sidak's multiple comparison test for the rotarod and beam walk tests with GraphPad Prism version 6.00 for Windows, GraphPad Software (La Jolla, California, USA). The densitometric data from western blots were analyzed by one-way ANOVA with Fishers LSD test. The statistical analyses of other tests were done by Students unpaired two-tailed *t*-test. Unless specified otherwise, all data were expressed as mean ± SEM.

Results

Brain Fatty Acid Analysis

To verify the effect of the respective diets on the brain levels of fatty acids, we assessed cerebellar lipid composition on Day 7 after TBI (Table 2). The fatty acids that were most affected by the two diets were DHA and docosapentaenoic acid (DPA n-6) (Table 1). There was more than three-fold decrease in the DHA levels (from 15.1±2.1 to 3.9±1.0%) in the brain of mice fed with omega-3 deficient diet. As expected, the omega-3 deficient diet significantly increases brain DPAn-6 levels, from the undetectable level to 7.7±0.7%. Thus the brains of the mice fed with omega-3 deficient diet for three generations had substantial depletion in DHA, which was replaced by DPA as has been described earlier [24].

Effects of Brain DHA Status on Motor Function Recovery after TBI

There was a significant difference in the latency to fall off the rotarod between the two diet groups of mice after TBI (Figure 1A).

Table 2. Fatty acid analysis using the cerebellum of TBI mice on omega-3 fatty acid adequate or deficient diet *(n = 4)*.

Fatty Acid Content (weight %, mean ± SD)		
	Adequate	**Deficient**
16:0	18.4±1.1	18.0±0.8
18:0	20.6±0.7	20.3±0.5
20:0	0.6±0.1	0.8±0.1
22:0	0.6±0.2	0.8±0.3
24:0	1.6±0.5	1.7±0.4
16:1	0.6±0.1	0.5±0.03*
18:1*n9*	20.4±1.7	19.0±1.0
18:1*n7*	5.0±0.2	5.6±0.2*
20:1*n9*	3.8±0.9	4.3±0.6
24:1	2.7±0.8	3.2±0.5
18:2*n6*	0.6±0.1	0.4±0.08*
20:3*n6*	2.2±0.3	2.2±0.2
20:4*n6*	6.2±0.4	8.1±0.5**
22:4*n6*	1.5±0.3	3.4±0.3***
22:5*n6*	0.0	7.7±0.7***
22:6*n3*	15.1±2.1	3.9±1.0***

*$p<0.05$, **$p<0.01$ and ***$p<0.001$ compared to the adequate diet group.

doi:10.1371/journal.pone.0086472.t002

The DHA deficient mice showed delayed recovery from the day 1 to day 7 after TBI ($p<0.01$) as calculated by repeated measures Two way ANOVA. Further analysis by applying Sidak's multiple comparisons test revealed significant differences on day 2 (adjusted $p<0.001$) and day 4 (adjusted $p<0.05$). The two diet groups had virtually identical latency periods (290.6 ±3.2 for adequate vs. 290.5±4.1 for deficient) when tested after three days of training (on the day before surgery), indicating that DHA depletion in the brain did not result in performance deficits in motor skills in naïve mice. The foot slips during the beam walk test were converted to percent of total steps and the data were analyzed by Two-way ANOVA. A highly significant difference was found between the two diet groups ($p<0.0001$) with the DHA deficient group showing more foot slips (Figure 1B). Sidak's test for multiple comparisons revealed statistical significance at all time points from Day 2 through Day 7 after surgery except for Day 3. The number of foot slips by both groups was virtually identical on the day before surgery (6.3±2.7 and 8.8±2.2 respectively).

Effects of Brain DHA Status on Anxiety-like Behavior and Cognitive Deficits after TBI

Anxiety-like behavior was studied by using the open field test and cognitive ability was assessed by the novel object recognition test. The time spent in the centre zone of an open field arena was used as to assess anxiety-like behavior. An unpaired two-tailed t-test yielded significant difference between the two diet groups, indicating that severe DHA deficiency leads to more anxiety-like behavior (63.7±10.0 vs. 22.9±4.3 for the omega-3 adequate group, $p<0.05$) (Figure 2A). This test was repeated for the TBI diet groups, which had similar differences in the time spent in the centre zone (28.7±6.2 vs. 11.1±3.1 for the DHA deficient groups, $p<0.05$) (Figure 2B).

The brain injured mice on omega-3 adequate diet had greater novel object exploration score than their deficient counterparts with a highly significant difference between the means (67.2±3.1 vs. 52.6±2.3 $p<0.01$) (Figure 2D). The non-injured mice did not show any difference between the two diet groups in the exploration time of the novel object, indicating that with our settings, the diets alone did not affect the ability of the mice to discriminate between familiar and novel objects (Figure 2C).

Spectrin-αII Breakdown and Synapsin-1 Expression

TBI-induced breakdown of alpha-spectrin or alpha-fodrin, a marker of TBI, was assessed by western blotting (Figure 3A). Spectrin breakdown products (SBDPs) were detected at 145 kDa and 150 kDa. Densitometric analysis indicated that TBI significantly increases SBDPs in both diet groups. The cerebral cortex from the adequate animals had more than 3 fold increased levels of SBDP after TBI ($p<0.05$) and the DHA depleted group showed an even greater (more than 6 fold) elevation ($p<0.01$ for 145 kDa product; $p<0.001$ for 150 kDa product). Consequently, injured cortices of the DHA deficient mice had a relatively higher increase of both 150 and 145 kDa fragments as compared to those of the adequate diet group ($p<0.05$) (Figure 3A). Synapsin-1 protein expression was about 30% less in the cortex of the contralateral hemisphere of the mice fed with omega-3 fatty acid deficient diet ($p<0.05$) compared to those on adequate diet, indicating that DHA deficiency is by itself sufficient to decrease synapsin-1 expression as reported earlier [25]. TBI resulted in the reduction of synapsin 1 in both diet groups with the synapsin level in the adequate and deficient groups at 60% ($p<0.001$) and 45% ($p<0.001$) of the respective controls. Despite the lack of statistical significance in the differences in synapsin 1 level between the injured diet groups, a decreasing trend was observed in the deficient TBI group (Figure 3B).

NeuN Immunostaining

Immunostaining with NeuN and subsequent quantification indicated substantial decrease in NeuN-positive cells in the peri-contusional area. The margins of the injured area were mostly devoid of specific staining and the NeuN staining gradually increased away from the injury site. The deficient group showed reduced NeuN positive cells (157.0±17.8) in comparison to the adequate group (222.0±21.4) ($p<0.05$) (Figure 4).

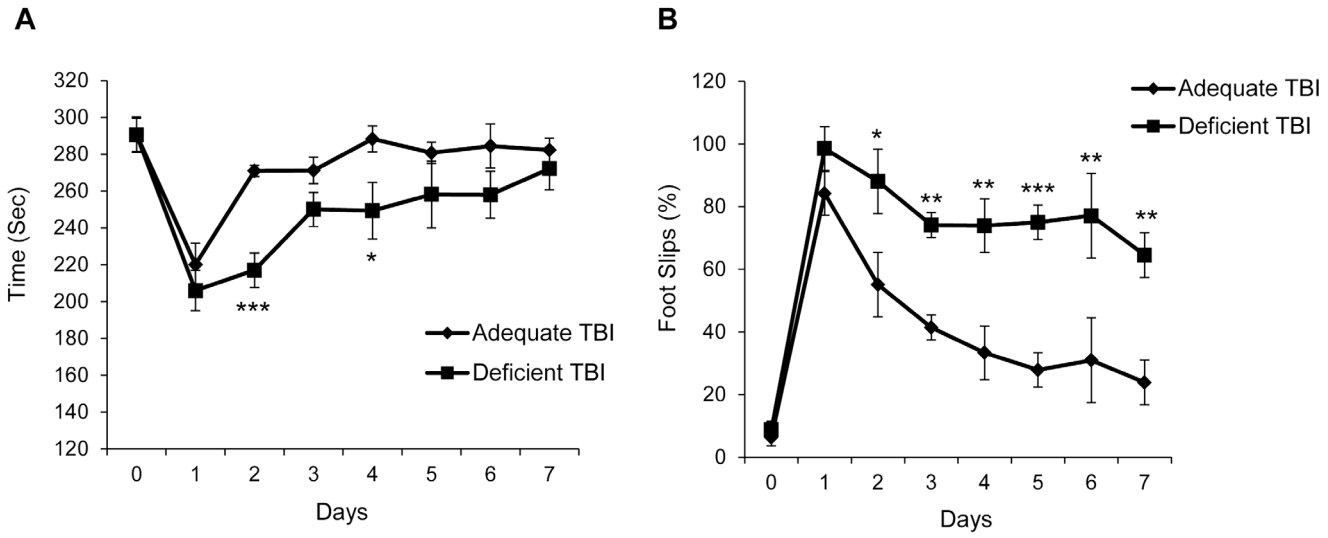

Figure 1. Omega-3 fatty acid deficiency impairs recovery from TBI-induced motor deficits. (A) The rotarod test showing slower recovery from TBI in the DHA deficient group (Deficient TBI) as compared to the adequate (Adequate TBI) mice with statistically significant differences on day 2 and day 4 after TBI (* $p<0.05$ and *** $p<0.001$ vs. the respective O-3 adequate group; $n=8$). (B) The beam walk test showing greater hindlimb footslips in DHA deficient mice as compared to the respective adequate controls (* $p<0.05$, ** $p<0.01$ and *** $p<0.001$ vs. adequate TBI group; $n=7$–8).

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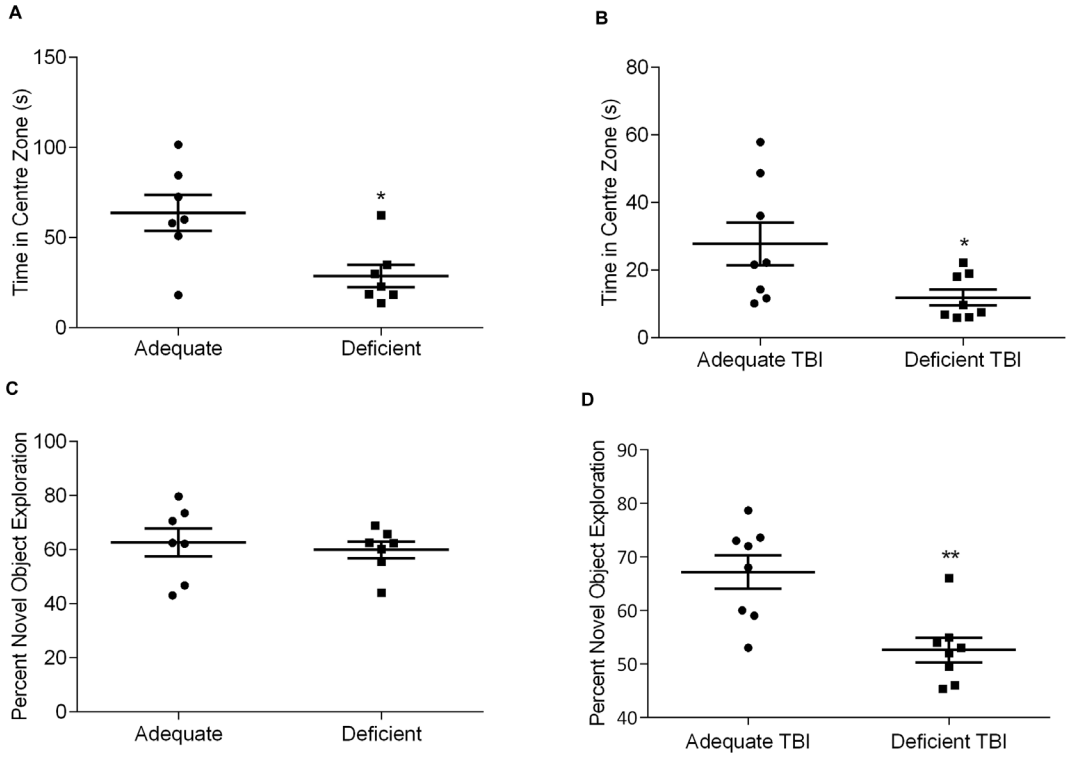

Figure 2. DHA depleted mice exhibit greater anxiety-like behavior and post-TBI memory deficits. (A) DHA deficient (Deficient) mice spend significantly less time in the centre zone of the open field than their DHA adequate (Adequate) counterparts (* $p<0.05$ vs. Adequate), thus exhibiting anxiety-like behavior. (B) A similar pattern of exploration observed on day 5 after TBI (* $p<0.05$ vs. Adequate TBI; $n=8$). (C) No significant difference observed in novel object exploration time between the non-injured DHA adequate and deficient mice ($n=7$). (D) Significantly less exploration of the novel objects in DHA depleted mice on day 7 after TBI than their adequate counterparts (** $p<0.01$ vs. Adequate TBI; $n=8$).
doi:10.1371/journal.pone.0086472.g002

TBI Lesion volume

TBI by controlled cortical impact resulted in distinct cavitation in the injured cerebral hemisphere (Figure 5A). Although the DHA-depleted animals showed an increasing trend of the lesion volume (8.24 mm3±0.8 SEM and 9.83±1.06 SEM for DHA adequate and DHA depleted TBI mice respectively), the difference was not statistically significant (Figure 5B).

Discussion

The present study demonstrates that i) severe DHA deficiency in the brain impairs functional recovery from TBI in terms of vestibulo-motor and cognitive deficits (ii) DHA deficiency further elevates TBI-induced production of SBDPs (iii) less neurons were found around the injury site of DHA deficient brain after TBI compared to the omega-3 fatty acid adequate group. Little is known regarding the effect of brain omega-3 fatty acid depletion on the predisposition toward altered recovery from TBI. Studies report that omega-3 fatty acid deficiency for a single generation can only decrease the DHA content by up to 40% whereas severe DHA depletion (up to 80%) can be achieved after two or more consecutive generations [26]. Over 70% of DHA depletion was observed in the cerebellum by feeding animals with omega-3 fatty acid deficient diet for three generations in the present study. It should be noted that the fatty acid analysis was performed for cerebellum, a part that has a vital role in the control and coordination of vestibulo-motor activity. We presume that the change in DHA levels in the cortex would be similar to that in the cerebellum. Indeed, the change in DHA level in the cerebellum in rats reared on omega-3 fatty acid deficient diet for two successive generations is comparable to that in the cortex [27]. Improved motor performance has been observed after focal cerebral ischemia [14] and neonatal hypoxic-ischemic brain injury [28] and spinal cord injury [29] after treatment with omega-3 fatty acids and in peripheral nerve injury [30] in fat-1 transgenic mice that have elevated endogenous omega-3 fatty acid level. The present study shows an improvement in the vestibulo-motor function in TBI mice by employing two motor tests. The rotarod test, particularly the accelerated version of the rotarod test, is a sensitive test to evaluate TBI induced motor deficits [19]. Our results indicate that recovery from acute motor deficits is impeded in the DHA deficient mice, which have shorter latencies on the rotarod. The beam walk test has been extensively used to detect differences in fine motor coordination. The difference in spontaneous recovery assessed by this test is even more pronounced than that evaluated by the rotarod test with the deficient group having more than 50% foot slips after a week from injury. These findings indicate that DHA deficiency interferes with the recovery of vestibulo-motor function after trauma. Incidentally, two studies were published during the preparation of this manuscript that deal with the effect of supplementation or depletion of omega-3 fatty acids/DHA on the outcome after cortical impact injury. Pu et al. have found improved functional recovery and reduced white matter damage after cortical impact injury in mice supplemented with omega-3 fatty acids for two months [31]. Similarly, in a study employing age-specific rat juvenile TBI model, Russell et al. found worsened motor deficits after TBI in DHA-depleted juvenile rats as compared to those on

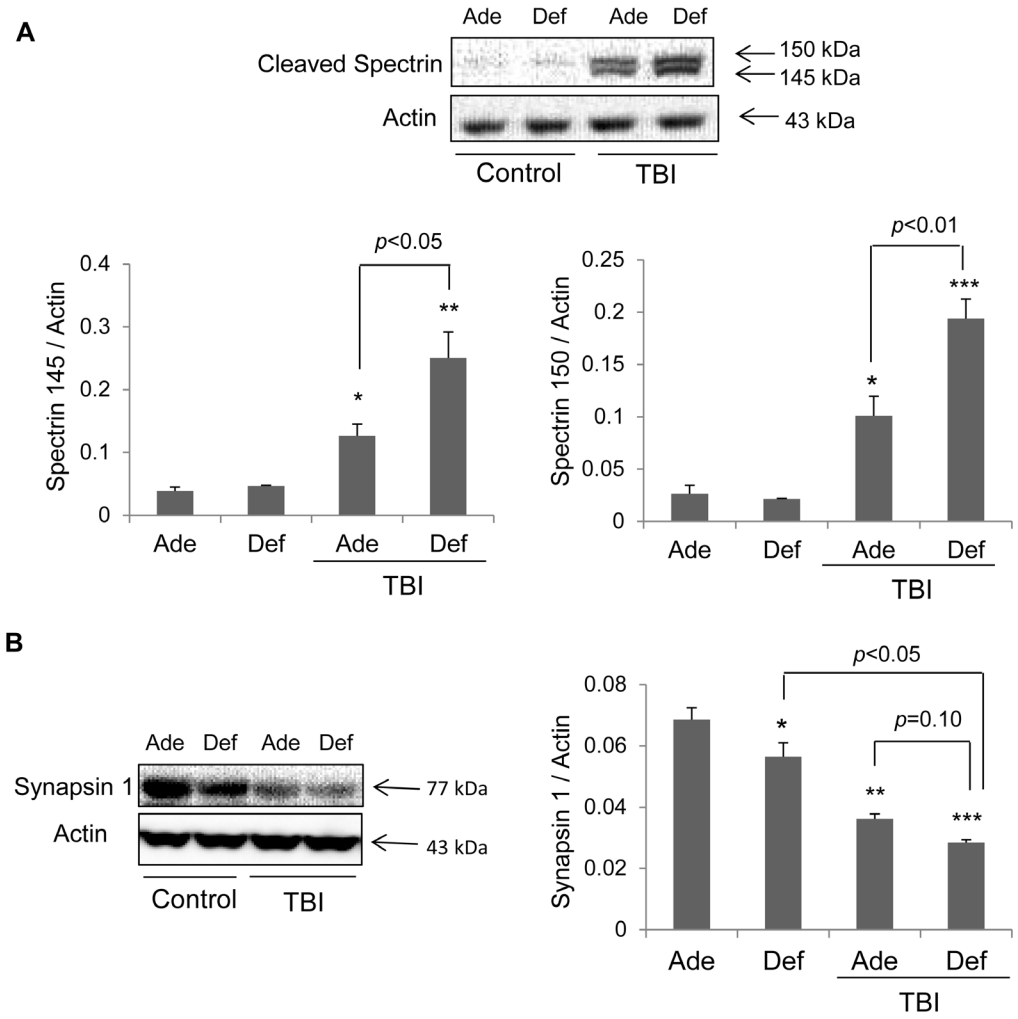

Figure 3. DHA deficiency increases TBI-induced spectrin-αII breakdown products (SBDPs) and decreases synapsin 1. (A) SBDP levels differentially increased after TBI in the affected cortices of DHA adequate (Ade) and deficient mice (Def). The contralateral cortices were used as controls. The deficient TBI group showed a significantly greater increase in SBDP levels after TBI for both 145 kDa and 150 kDa fragments as compared to the adequate TBI group ($n = 4$). (B) Synapsin 1 level affected by TBI and DHA depletion. TBI significantly decreased the synapsin-1 level in both diet groups. DHA depletion lowered the synapsin significantly ($p<0.05$) in the non-injured mouse cortices. Although statistical significance was not reached, a trend of further decrease in synapsin 1 level after TBI was observed with DHA deficiency. * $p<0.05$; ** $p<0.01$; *** $p<0.001$ compared to the non-injured adequate group.
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the control diet [32]. This is consistent with our findings in that DHA depletion in the brain by chronic omega-3 fatty acid deficiency exacerbates the injury and impedes the functional recovery.

The effect of deficiency of omega-3 fatty acids on anxiety and memory impairment has been extensively studied. There have been mixed reports regarding the effect of omega-3 deficiency on anxiety. Takeuchi et al. found that omega-3 deficient rats exhibit greater anxiety-like behavior in the elevated plus maze that was reversed by DHA supplementation [33]. In contrast, no significant differences were found by Nakashima and colleagues [34]. Carrie et al. showed that although omega-3 deficient mice showed greater anxiety in the open field, it was only partially alleviated on reversing the deficiency [35]. We found significant decrease in the time spent in the centre zone by DHA deficient mice for both sham and TBI groups indicating that the basal anxiety differences are due to DHA depletion and are maintained after trauma.

Many studies have demonstrated decrease in cognitive functions as a result of omega-3 fatty acid deficiency created through differential dietary regimens. In an experimental setup similar to the present study, Moriguchi et al. have reported that omega-3 fatty acid deficiency has a "dose dependent" impact on spatial memory impairment assessed by the Morris water maze test with the third consecutive generation of rats reared on omega-3 deficient diet showing greater deficits in memory as compared to the second generation [24]. Moreover, in a subsequent paper, the authors reported that such omega-3 fatty acid deficiency induced memory impairment is reversible and can be corrected on omega-3 replenishment [36]. We did not find significant changes in object recognition memory in the sham diet groups (Figure 2C). This may be attributed to two causes. One reason may be that the novel object recognition test assesses non-spatial object recognition. The second possible reason is that the duration of training for the novel object recognition test in the present study may be long enough to conceal any subtle cognitive deficits due to DHA depletion alone.

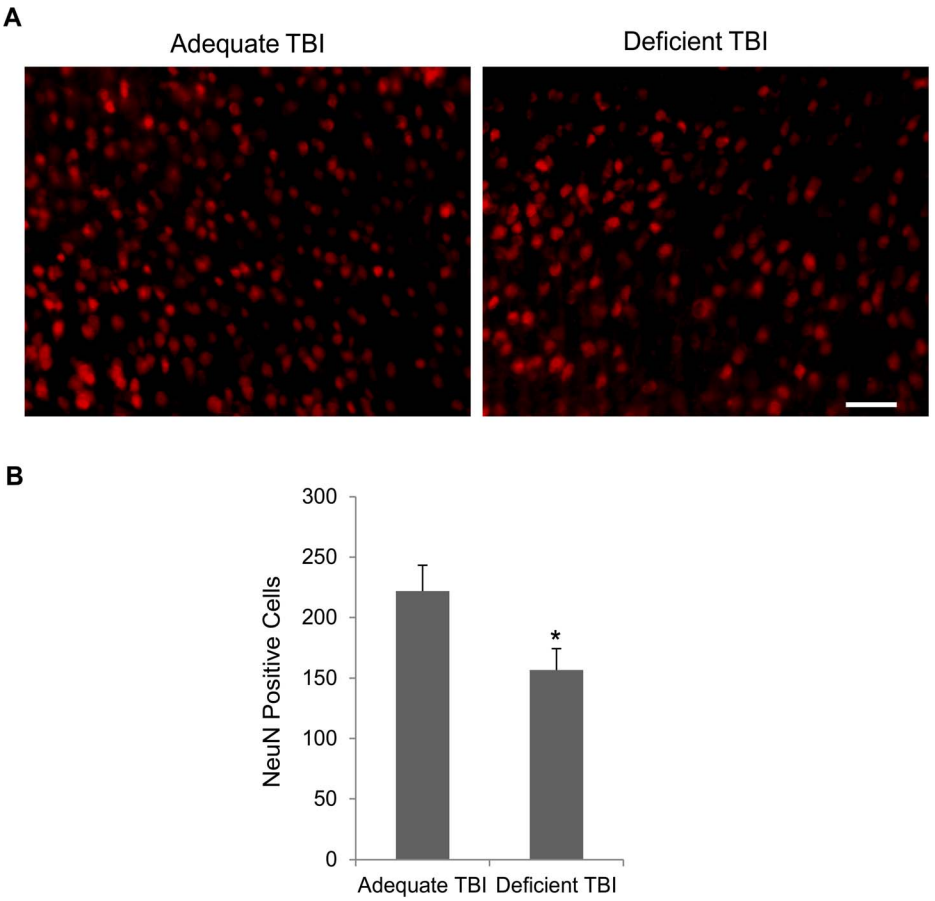

Figure 4. DHA deficiency decreases NeuN positive cells after TBI. DHA depleted mice showed decreased NeuN positive cells in the pericontusional cortices relative to the DHA adequate mice as indicated by the representative micrographs (A) and quantitative analysis (B) of 25 brain sections from 5 mice. Scale bar = 50μm. * $p<0.05$.
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Nevertheless, the changes in percent exploration of the novel object after injury are significantly different between the diet groups (Figure 2D). Also, for the mice on omega-3 fatty acid adequate diet, the percent novel object exploration score of the sham mice is comparable to that of the TBI mice. We believe that the main reason for this is the long exploration time during the training phase, which was up to 15 min, coupled to a shorter interval before the test phase, as opposed to the shorter training durations of 5 min generally used for this paradigm [37]. Thus the inability of the DHA deficient TBI mice to recognize the novel object despite the extended training emphasizes compromise in cognition in this group. Furthermore, DHA in the adequate diet, even though it was present in low amount, may also contribute to protection from cognitive impairment after TBI.

Membrane DHA in the brain provides resilience against propagation of injury-induced cellular damage and facilitates adaptive responses for recovery that are not adequately compensated for by omega-6 DPA that replaces DHA in the brains of mice on omega-3 fatty acid deficient diet. Indeed, naïve rats that were artificially hand fed with linoleic acid supplemented with DPA during infancy and later with similar pellets had correspondingly lower brain DHA levels and defective spatial retention as compared to the DHA-supplemented rats [38]. It is also possible that the increase in free fatty acids due to activation of phospholipases after TBI is a contributing factor in promoting recovery. A localized increase in free fatty acids at the site of injury has already been reported [39,40]. In this case, DHA deficient mice would be expected to release less free DHA that can be converted to neuroprotective, anti-inflammatory and proresolving mediators such as neuroprotectin D1 or resolvins [41], which may result in increased susceptibility of neurons and greater neuronal loss encountered in this study. In addition, low free DHA would limit the production of synaptamide (docosahexaenoylethanolamide), a neurogenic, neuritogenic and synaptogenic metabolite of DHA [42,43] that may have a bearing on functional recovery from trauma.

Non-erythroid alpha-spectrin II is a neuronal cytoskeletal protein that is a substrate for calcium activated cystein proteases, calpains and caspase-3. 145 kDa and 150 kDa fragments of alpha-spectrin II are remain elevated after severe TBI for 48 hr [44]. The breakdown products of alpha-spectrin II have been proposed as a reliable marker for brain injury [45] and to predict the severity of injury in severe TBI [46]. In this study, TBI in the DHA deficient mice resulted in greater cortical SBDPs, implying more tissue damage occurred in the deficient brain. Also, increased spectrin break down may be interpreted as increased activation of these cystein proteases after injury in the omega-3 deficient mice. Wu et al. found that DHA supplementation in diet alleviates the decrease in CAMKII, a substrate of calpains, due to fluid percussion injury [11]. It is possible that omega-3 fatty acids may be able to modulate injury processes by regulating calcium activated pathways. Indeed, omega-3 fatty acids have been

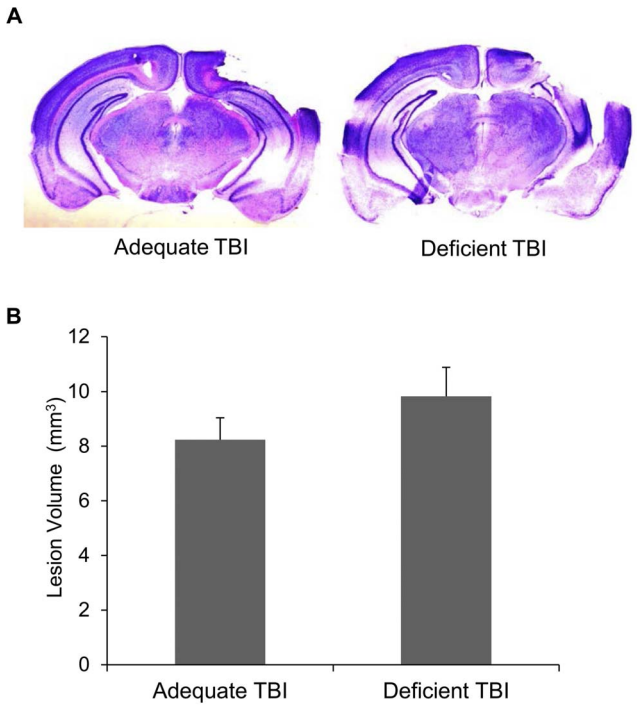

Figure 5. Depletion of DHA does not affect the volume of the lesion induced by TBI Lesion volumes of DHA depleted brains after TBI are not statistically different from those of the DHA adequate controls ($n = 6$).
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reported to inhibit native T-type calcium ion channels [47]. Synapsin 1, a phosphoprotein enriched in synapses and implicated in synaptogenesis [48], was found to be significantly reduced after TBI in both DHA adequate and deficient mice, suggesting that TBI induces loss of synapses. The decreasing trend of the synapsin level in deficient brains also suggests a role of DHA in sustaining synapses after TBI.

Membrane changes due to DHA depletion may result in increased susceptibility of neurons to cell death after injury, thereby leading to greater decrease in NeuN positive cells in these mice. DHA as a membrane component has been reported to be a positive modulator of neuronal survival by promoting Akt mediated survival signaling [49]. Chronic dietary DHA modulation decreases neuronal damage by decreasing post-ischemic inflammation [50]. The decrease in the number of NeuN positive cells after TBI in this study is likely to involve similar mechanisms. However, the decrease in brain DHA level did not reflect as increase in tissue loss after TBI. The lack of statistically significant change in the frank lesion volume may be because the gross estimation of lesion volume did not have adequate sensitivity to detect small differences coupled with modest sample number used for this analysis. However, these results are similar to Pu and colleagues' findings of relative increase in neurons in hippocampal CA3 in the omega-3 fatty acid (DHA/EPA) supplemented TBI mice without any change in the lesion volume [31] and also to Russell et al.'s finding of lack of change in lesion volume in DHA depleted juvenile rats after TBI [32]. Thus, although DHA/omega-3 fatty acids seem to effectively reduce infarction after cerebral ischemia [14,15,28], they may not affect frank lesion volume after TBI [31,32].

In summary, severe depletion of membrane DHA in the brain renders mice significantly more susceptible to TBI and impairs recovery following the injury. Omega-3 fatty acids may serve as nutraceutical agents and precondition the brain to make it more resilient to injury. From this data, it can be suggested that enriching DHA in the brain may be prophylactic and protective against brain injury. Further studies with acute administration of DHA or its metabolites are needed to explore the possibility of its use at the therapeutic level.

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Author Contributions

Conceived and designed the experiments: HYK AD. Performed the experiments: AD KK. Analyzed the data: HYK AD. Wrote the paper: HYK AD.

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Neuroprotection by Docosahexaenoic Acid in Brain Injury

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ABSTRACT Docosahexaenoic acid (DHA, 22:6 n-3) is an omega-3 polyunsaturated fatty acid highly enriched in the brain and is recognized as an essential nutrient for proper development of brain function. Common brain injuries often cause lifelong neurological and cognitive impairments, especially in learning and memory. Optimizing the nutritional DHA status in neural tissues may allow significantly improved resilience for the central nervous system to injury and optimized recovery. This article discusses neuroprotective effects of DHA, which are potentially important for improving injury outcome, thus reducing the risk of lifelong neurological impairment associated with brain injury.

INTRODUCTION

Numerous studies have shown that docosahexaenoic acid (DHA) is essential for proper brain development and function,[1,2] although the underlying mechanisms are still unfolding. Under normal conditions, DHA is present in esterified form in membrane phospholipids, especially the aminophospholipids, phosphatidylethanolamine, and phosphatidylserine (PS). Despite tight regulation to maintain membrane phospholipid homeostasis, DHA enrichment can expand the PS pool in the neuronal membranes,[3] as DHA-containing phospholipids serve as the most favored substrate for PS biosynthesis in mammalian tissues.[4] On the contrary, depletion of DHA has been shown to decrease PS levels significantly in brain tissues.[3,5–7] Because PS is known to participate in key signaling events supporting cell survival and differentiation, DHA-dependent PS modulation is an important aspect of neuroprotection.[8] Following brain injury, polyunsaturated fatty acids (PUFAs) including DHA and arachidonic acid (AA; 20:4 n-6) are released from neural membranes and metabolized to many bioactive derivatives. Some of the AA-derived eicosanoids are known to be proinflammatory, exacerbating the initial injury.[9,10] In contrast, some DHA-derived docosanoids has been shown to ameliorate or resolve inflammatory processes.[11] Furthermore, a DHA metabolite of a separate class has been recently identified as a potent neuritogenic, synaptogenic,[12] and neurogenic agent.[13] In this regard, DHA content in the brain may be an important variable to consider in devising a strategy to improve neuroprotection and recovery outcome after brain injuries.

DISCUSSION

DHA Enrichment in the Brain and Neural Resilience

The DHA-induced PS increase in neuronal membranes can prevent neuronal apoptosis under challenged conditions, and thus promote neuronal survival at least in part through facilitating signal transduction of key kinases such as PKC, Raf-1, and Akt.[8,14–16] The interaction of PKC with membrane PS for its activation is well established.[17] Likewise, Raf-1 activation also requires interaction with acidic phospholipids including PS and phosphatidic acid.[18,19] Akt activation is known to be dependent on the PIP_3 generation. However, interaction between Akt and membrane PS is also recognized to be crucial for Akt activation,[20] providing a molecular basis for DHA-derived improvement of neuronal survival, especially under PIP_3-limited conditions. Considering the difficulties inherent in neuronal cell regeneration, improved neural resilience because of PS-dependent prosurvival signaling is a significant mechanism for maintaining optimal brain function.

The DHA status in the brain is known to be influenced by the diet. Although the dietary supply of DHA improves cognitive development in humans,[21,22] and memory-related learning ability in rats,[23] diets deficient in omega-3 fatty acids (FAs) deplete brain DHA and impair hippocampus-related functions such as learning and memory.[24–26] Varying degrees of DHA deficiency can be induced according to the omega-3 FA content in the diet relative to the omega-6 FA content as well as the duration on such diet.[24,27,28] For example, the DHA level in the hippocampus of offspring mice at 3 weeks of age decreased from 12.6% to 3.3% when the pregnant mother was fed with either an omega-3 FA-adequate or omega-3 FA-deficient diet throughout the pregnancy and lactation period (Table I). This decrease in DHA in the hippocampus resulted in the impairment of long-term potentiation (LTP),[29] an established cellular model for learning and memory (Fig. 1).[30] Concomitantly, there was a significant reduction in hippocampal expression of N-methyl-D-aspartate (NMDA) receptor subunits and synapsins,[29,31] which are known to be critical for LTP as well as learning and memory.[30,32]

At the cellular level, DHA deficiency caused retarded hippocampal neuronal development (Fig. 2), and increased susceptibility to neuronal cell death under challenged conditions (Fig. 3).[16] These findings suggest that it is important to maintain the optimum DHA status by dietary omega-3 FA intake for neuronal protection, particularly under stressful conditions, and for proper cognitive function.

Brain DHA Status and Recovery After Brain Injury

Long-term omega-3 FA intake, which increases DHA in the brain, has been shown to be beneficial in many pathophysiological settings. For example, DHA supplementation has alleviated symptoms in some patients with peroxisomal disorders,[33] prevented dendritic pathology[34] and impairment of

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TABLE I. Effect of Dietary Depletion of *n*-3 Fatty Acids on Hippocampal Fatty Acid Composition at 3 Weeks of Age

	Fatty Acid Content (Average wt% ± SD)	
	n-3 Adequate Diet	n-3 Deficient Diet
20:3 n-6	0.64 ± 0.06	0.29 ± 0.04
20:4 n-6	12.34 ± 0.29	13.89 ± 1.00
22:4 n-6	1.98 ± 0.17	3.28 ± 0.25*
22:5 n-6	0.30 ± 0.03	8.55 ± 1.24*
Σn-6 Polyunsaturates	15.26	26.01
22:5 n-3	0.19 ± 0.06	<0.05*
22:6 n-3	12.39 ± 0.99	3.33 ± 1.50*
Σn-3 Polyunsaturates	12.58	3.31
n-6/n-3 Ratio	1.21	7.86

*$p < 0.001$ compared to the adequate group.

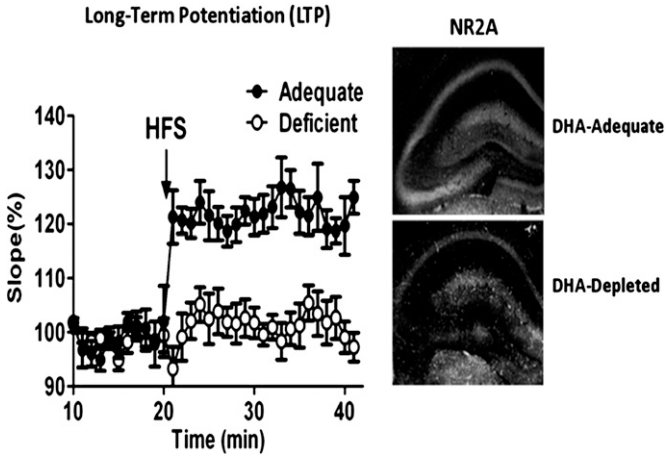

FIGURE 1. Inhibition of LTP induced by high frequency stimulation (HFS) and reduced NR2A expression in the mouse hippocampi (3 weeks of age) because of DHA depletion caused by omega-3-deficient diet.[24]

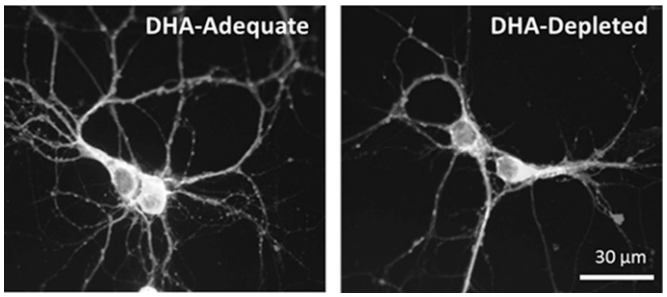

FIGURE 2. Hippocampal neurite growth and synaptogenesis (evaluated by synapsin puncta) impaired by DHA depletion.[24]

learning ability[35] in rodent models of Alzheimer's disease, and protected dopaminergic neurons from toxin-induced degeneration in a mouse model of Parkinson's disease.[36] Conversely, low DHA levels in the brain is associated with neuropathological conditions in humans, such as generalized peroxisomal disorders[37] and Alzheimer's disease.[38] Multiple mechanisms can be considered for the neuroprotective effects of DHA after injury; reduction of oxidative stress by increasing glutathione reductase activity[35] or decreasing reactive oxygen species (ROS) generation associated with injury[35,39]; membrane modification that in turn can modulate ion channel activities,[40,41] receptor-mediated signal transduction[8] and capacity to generate lipid mediators[42,43]; or alteration of gene transcription[44] possibly thorough activation of nuclear receptors such as retinoid X receptor (RXR),[45] to list a few. These mechanisms, working separately or in concert, contribute to DHA's neuroprotection, alleviating toxicity and injury propagation while facilitating repair processes.

The sequel of the brain injury often involves the release of PUFAs from the neural membranes, microglia activation, and recruitment of leukocytes into brain tissues following the initial tear and shear of tissues and hemorrhage. The released AA (20:4 n-6) is converted by cyclooxygenase and lipoxygenase enzymes to two-series prostaglandins/thromboxanes and leukotrienes, respectively. Many of these AA-derived metabolites are proinflammatory mediators, increasing leukocyte infiltration and proinflammatory cytokine production and furthering neurotoxicity.[9,10] Increasing tissue levels of DHA by dietary omega-3 FA supplementation or DHA pretreatment has been shown to significantly improve ischemia/reperfusion injury outcome measures such as cerebral infarct area,[46–48] cerebral edema,[48] and blood flow[48] as well as neuronal survival.[49–51] Recently, dietary supplementation with DHA before traumatic brain injury (TBI) has been found effective in reducing axonal injury.[52] Decreases in tissue AA and its inflammatory metabolites also have been observed in DHA-treated animals,[48,53] suggesting that neuroprotection by DHA in ischemia/reperfusion injury may be partly because of lowering AA levels in brain tissues. Considering that the activation of oxidative metabolic enzymes together with mitochondrial dysfunction is considered a significant cause for ROS generation after injury,[54] lowering AA and thus cyclooxygenase- or lipoxygenase-dependent AA metabolism with DHA may reduce ROS-related injury propagation. DHA released during injury can also be converted to eicosanoid-like metabolites such as resolvins that ameliorate or resolve inflammatory processes,[11,42] in contrast to the proinflammatory action of many eicosanoids derived from AA.[10,55] Neuroprotectin D1 (10R,17S-dihydroxy-docosatriene, NPD1),[56] another DHA metabolite generated under pathological conditions or after injury, was shown to be effective in preventing neuronal cell death. Therefore, enriching DHA and simultaneously lowering AA in the brain through manipulation of the omega-6 to omega-3 ratio in the diet may be of critical importance in improving the outcome of brain injury.

Neurite elongation, dendritic arborization, and synapse formation also are important reparative phenomena after injury, although not all neurons are able to spontaneously initiate this repair process. It has been reported that spontaneous extensive remodeling of central nerve circuits occurs after an injury, evidenced by axonal sprout formation and removal, which may play an important role in recovery.[57,58] We have demonstrated that DHA uniquely promotes elongation and branched growth of neurites at low micromolar

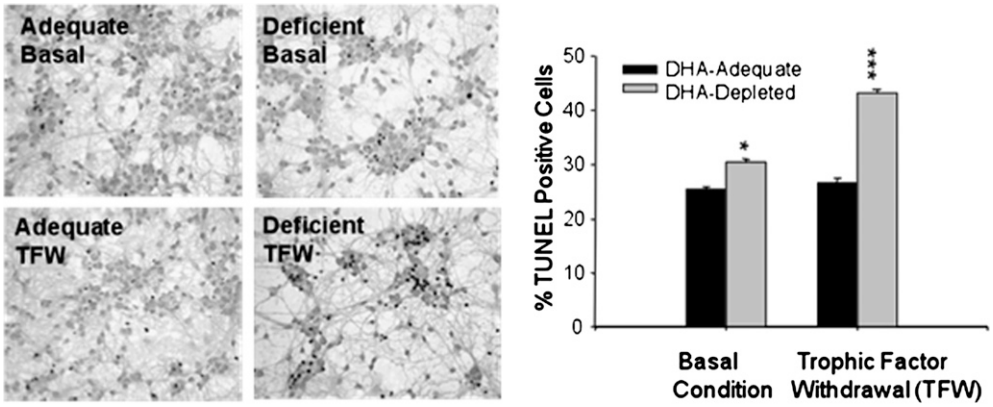

FIGURE 3. Increased susceptibility to cell death in embryonic neuronal cultures from DHA-depleted fetal hippocampi because of maternal dietary deficiency of omega-3 fatty acids.[10] Cell death was evaluated by TUNEL staining shown as black dots in the micrographs. TFW, trophic factor withdrawn condition; TUNEL, Terminal deoxynucleotidyl transferase dUTP nick end labeling.

concentrations in developing hippocampal neurons.[29,59] Synapsins, a family of neuron-specific phosphoproteins associated with the membranes of synaptic vesicles, have been identified as a molecular component involved in synaptogenesis, synapse maturation, and synaptic function.[60–62] With DHA treatment, hippocampal neurons showed a significantly increased number of synapsin-positive puncta normalized per neuron or neurite length, suggesting improved synaptogenesis in developing neurons (Fig.4).[29] The excitatory synaptic activity involved in learning and memory was also improved in DHA-enriched neurons.[29] Recently, *N*-docosahexaenoyletha nolamine, an amide form of DHA metabolite synthesized in neuronal cells, has been demonstrated to be a potent mediator for DHA-induced neuritogenesis, synaptogenesis,[12] and neurogenic differentiation.[13] The term "synaptamide" was coined for this metabolite, based on the endocannabinoid-like amide

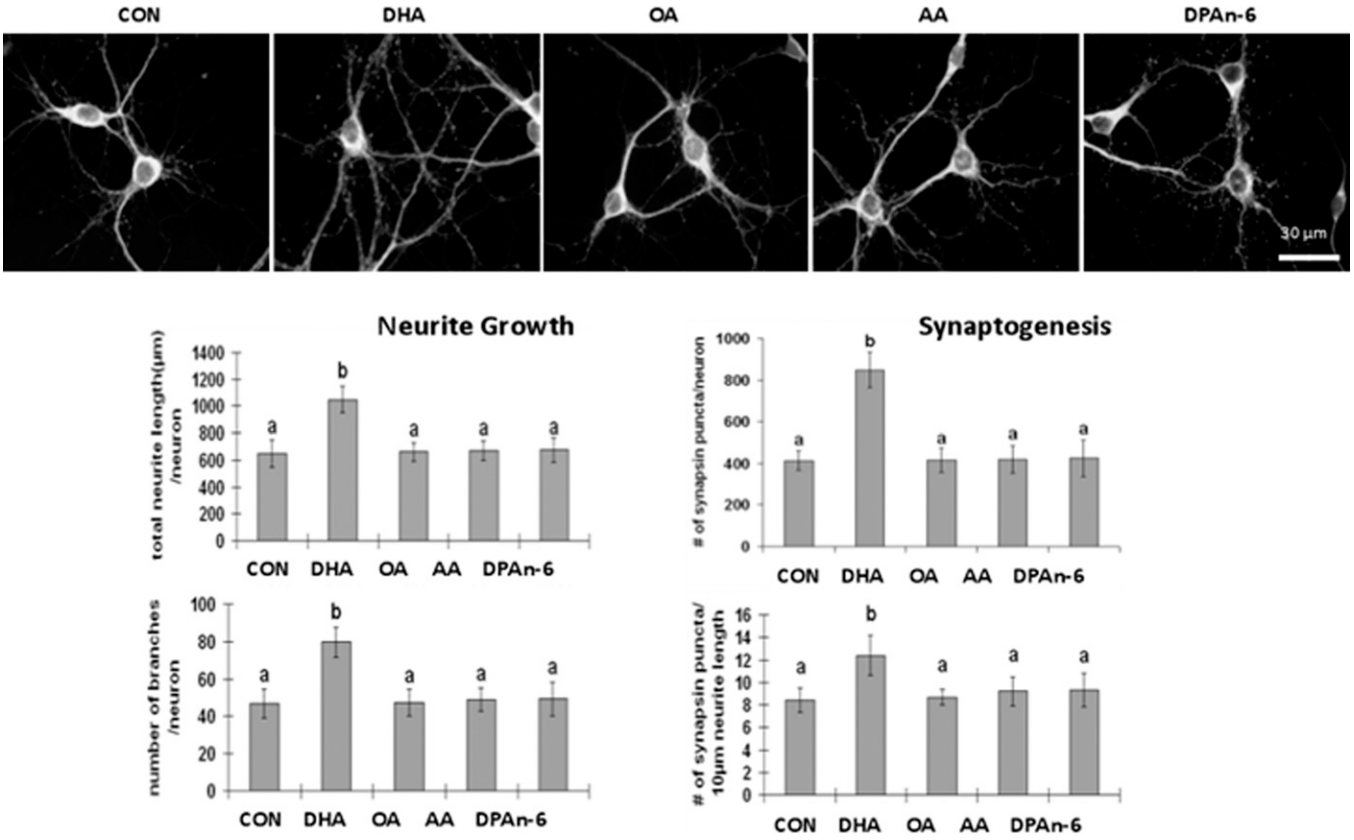

FIGURE 4. Hippocampal neurite growth and synaptogenesis (evaluated by synapsin puncta) promoted uniquely by DHA supplementation.[24] The cultures were supplemented with 1 µM for 10 days. Different alphabetical letters indicate significant differences at $p < 0.05$. OA, oleic acid (18:1 n-9); DPAn-6, docosapentaenoic acid (22:5 n-6).

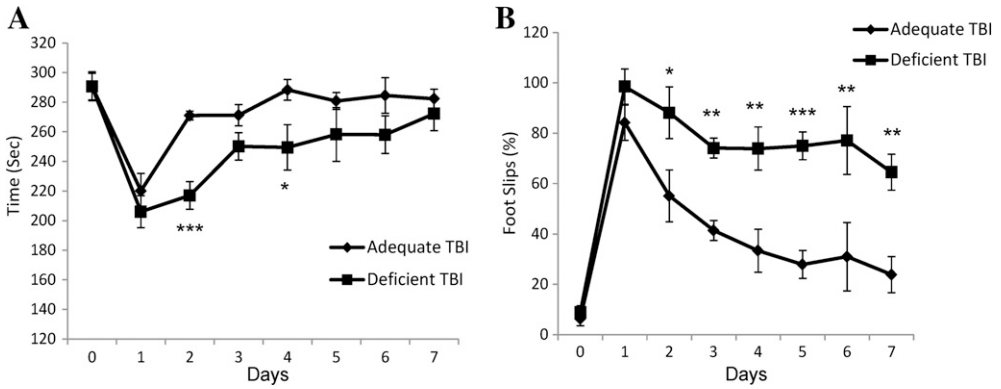

FIGURE 5. Spontaneous recovery from TBI-induced motor deficits impaired after reduction of brain DHA by omega-3 deficient diet.[62] Mice were kept on an omega-3-adequate or omega-3-deficient diet for two generations and TBI was inflicted by controlled cortical impact (CCI) at the age of 3–4 months. Recovery from TBI-induced motor deficits evaluated by (A) rotarod or (B) beam walk test was significantly slower for the DHA-deficient group (Deficient TBI) as compared to the adequate (Adequate TBI) mice.

structure and potent synaptogenic property.[63,64] Endogenous levels of synaptamide and DHA in the brain were shown to be directly linked.[12,65] Neurite elongation is normally limited to developmental neuritogenesis; however, significant changes in dendritic arborization occur beyond the developmental period and throughout life span,[66] and are known to be associated with structural plasticity.[67] In addition to neurogenesis, synaptamide may induce neurite elongation for regeneration of severed neurites and promoting dendritic arborization particularly in uninjured neurons, facilitating the neuronal repair and potentiate collateral reinnervation and synaptic reorganization required for functional recovery after injury. Therefore, increasing DHA in neural membranes, which is the reservoir for DHA release and subsequent metabolism, may be critically important for stimulating spontaneous regeneration of neural networks. In this context, neural DHA enrichment by long-term dietary means is prophylactic against injury and can improve injury outcome, including learning and memory function. Indeed, recent study indicated that lowering the brain DHA status by omega-3 FA-deficient diet was found to exacerbate TBI-induced neuronal cell death and impede functional recovery (Fig. 5).[68]

DHA's Therapeutic Potential in Brain Injury

In addition to the prophylactic advantage of neural DHA enrichment, the treatment potential of DHA following brain injuries also has been explored in recent years. For example, DHA infusion after 3 hours of experimental brain ischemia reduced neuronal injury,[69,70] and the administration of DHA

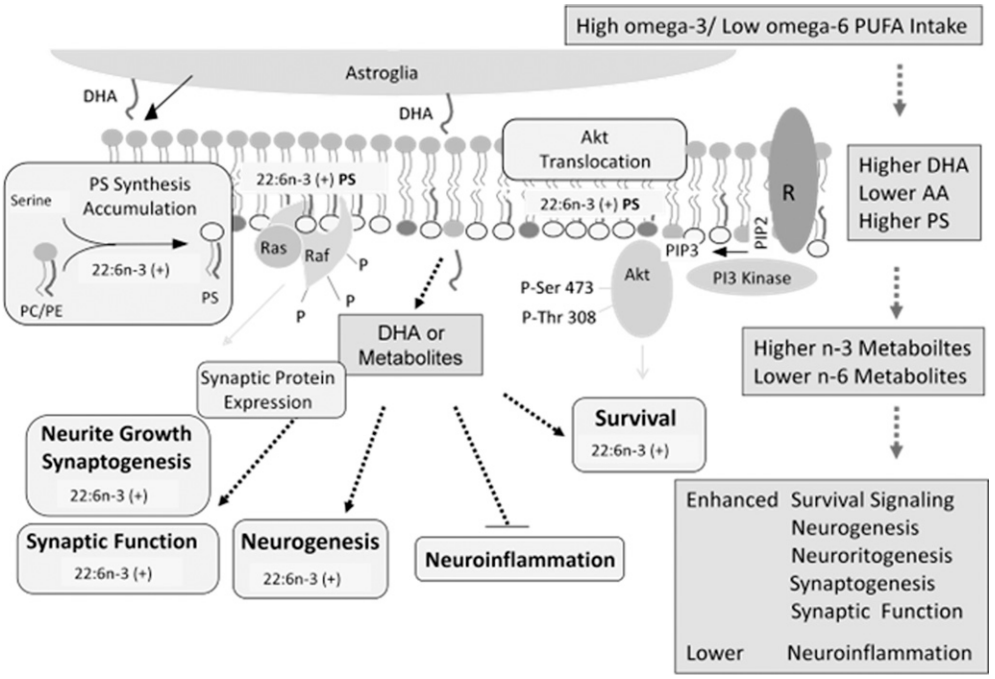

FIGURE 6. Mechanisms underlying neuroprotective effects of DHA.

or fish oil for 30 days following head injury improved traumatic axonal injury in a rat model.[71,72] Furthermore, a single injection of DHA at 1 hour after spinal cord injury markedly improved functional recovery in a rodent model.[73] The positive effects of DHA and its endogenous metabolite synaptamide on neurogenesis,[13] hippocampal neurite growth,[12,29,59] synapse formation, and synaptic function[29] also support DHA as a possible agent for stimulating the neuronal repair and reducing neurological impairment and cognitive deficits usually associated with brain injuries. Although limited, the evidence presented thus far suggests promising therapeutic potential for DHA and its metabolites in the treatment of brain injuries.

CONCLUSION

The evidence suggests that DHA provides "nutritional armor" for common brain injuries through suppression of the propagation of injury responses, prevention of neuronal cell death, and promoting neurogenesis and restoration of neurites, synapses, and synaptic function. Some of the basic mechanisms involved in the neuroprotective effects of DHA are summarized in Figure 6. Increasing dietary omega-3 FAs and lowering omega-6 FA intake for a prolonged period leads to higher levels of DHA and PS in brain tissues. Membrane-related events such as PS-dependent Akt, Raf-1, and PKC signaling are facilitated, which in turn enhances neuronal survival, particularly under suboptimal conditions. Although changes in AA levels may take longer, its proportion with regard to omega-3 FAs in neural membranes decreases, and so does the probability to generate AA-derived proinflamatory mediators. Prosurvival, anti-inflammatory, proneuroregenerative, and synapse enhancing properties of DHA-derived metabolites, which are generated during the course of injury progression, can also significantly contribute to neuroprotection by DHA. These mechanisms may provide insight for devising new prevention and treatment strategies for brain injuries.

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